Gas exchange under water

Acclimation of terrestrial plants to submergence

Gasuitwisseling onder water

Aanpassingen van landplanten aan overstroming

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Acclimation of terrestrial plants to submergence

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Chapter 1

General Introduction

Flooding is characterized as a highly dynamic process with an infrequent and unpredictable nature (Vervuren et al. 2003), but with structuring properties affecting plant species richness and species distribution (Silvertown et al. 1999, van Eck et al. 2004). Flooding results in clear distribution limits of plant species along the vertical elevation gradient of river floodplains (Sýkora et al. 1988, Lenssen et al. 1999, van Eck et al. 2004). Most flooding-sensitive species are restricted to high elevations of the floodplain gradient where floods are rare, while some others occur at lower sites but avoid flooding through life history tactics and survive unfavourable conditions as dormant seeds or perennial organs (Menges and Waller 1983, Van der Sman et al. 1993). Most species growing at low floodplain elevations, however, are tolerant to flooding. Depth and duration of a flood are the main environmental variables determining these distinct distribution patterns of plant species (Klimešová 1994, Blom and Voesenek 1996, Toner and Keddy 1997, Van de Steeg and Blom 1998, Casanova and Brock 2000).

Negative effects of submergence on plant performance

Complete submergence negatively affects plant growth and survival, since it severely inhibits gas exchange between the plant and the environment due to a 10^4 times lower diffusion rate of gases in water than in air (Armstrong 1979). As a result of the hampered gas exchange under water, oxygen concentrations within plants may decrease rapidly upon submergence (Stünzi and Kende 1989, Rijnders et al. 2000). Such low oxygen levels will limit aerobic respiration and other essential oxygen dependent processes (Armstrong and Gaynard 1976, Laan et al. 1990). Anaerobic metabolic pathways, such as fermentation, might compensate the low ATP yield due to hampered aerobic metabolism (Perata and Alpi 1993, Gibbs and Greenway 2003). These pathways, however, are far less efficient than aerobic respiration and thus reduce the pool of carbohydrate reserves quickly (Laan and Blom 1990, Guglielminetti et al. 1997). Oxygen deficiency is, therefore, accompanied by energy and carbohydrate deficits.

Light quantity and quality are also different under water compared to the conditions above the water. Aquatic environments are generally described as shaded environments, since light is attenuated by surface reflection, back-scattering and absorption by water and

dissolved particles (Holmes and Klein 1987, Sand-Jensen 1989). This may particularly apply to river water, in which the load of suspended sediment is often extremely high, and thus light availability is extremely low. For example, median transmission is below 1% in a flood of 1 m depth, even at the lowest levels of suspended loads observed during flooding (Vervuren et al. 2003).

Responses of terrestrial plants to submergence

Submergence tolerance may be realized by different suites of traits that are considered to reduce the negative effects of submergence (Armstrong et al. 1994a, Vartapetian and Jackson 1997). Traits of plants tolerating oxygen deprivation include generation of energy via glycolysis and fermentation and reduction of energy requirements for maintenance (Perata and Alpi 1993, Greenway and Gibbs 2003). Many other traits of plants, however, are directed to avoidance of oxygen deficiency. These features may be constitutive, i.e. a trait is inherently expressed, or inducible, i.e. a trait is optimized in response to submergence. A well described example leading to restoring the contact with the air and thereby the gas exchange capacity, is elongation of the shoot (reviewed in Voesenek et al. 2004), which includes re-orientation of the leaves (hyponasty; Cox et al. 2003) and increased growth of the petiole and lamina (Voesenek et al. 2003a). Within-plant gas diffusion is enhanced by longitudinal air channels (aerenchyma) in shoot and roots (Visser et al. 1996a, Colmer 2003) and by development of a gas-tight barrier in the roots to prevent oxygen from being lost into the anaerobic soil (Colmer et al. 1998, Visser et al. 2000b).

Voesenek et al. (2004) showed that only a subset of flooding tolerant plant species inhabiting the floodplains is capable of significant shoot elongation upon submergence. These species generally inhabit poorly dehydrated sites, where water remains standing for a long period of the year. Under these conditions shoot elongation is thus an efficient solution to avoid oxygen deficiency. Many species, however, experience fully submerged conditions in which the water is too deep to reach the surface through shoot elongation. A very straightforward way to reduce the shortages of oxygen and carbohydrates under such conditions would be the continuation of photosynthesis under water. As photosynthesis produces both oxygen and carbohydrates, it might alleviate submergence-stress in completely submerged plants considerably. Continuation of photosynthesis underwater is, however, a poorly investigated process in terrestrial plants.

Underwater photosynthesis in terrestrial plants

During photosynthesis oxygen and carbohydrates are produced from water and CO_2, in the presence of light as the energy source. The main process can be divided in three steps. 1). Light is absorbed by pigments, mainly chlorophylls, which transport the excitation energy to the reaction centers of the photosystems. 2). In the photosystems, the excitation energy splits water and simultaneously produces oxygen. The electrons that are derived from this process are transported over the electron transport chain and are accepted by electron acceptors. 3). The energy produced in the former step is used by the assimilation of CO_2 into carbohydrates. In C_3 photosynthesis, the carboxylation enzyme Rubisco binds CO_2 directly to its substrate ribulose-1,5- biphosphate (RuBP) in the first step of the carbon reduction cycle. CO_2 is, however, in competition with oxygen for binding sites at the Rubisco enzyme, and oxygen-bound Rubisco can catalyze the oxygenation of RuBP (Ogren 1984). These oxygenation reactions lead to photorespiration and thus to carbon losses.

The low light intensities that plants may experience during complete submergence limit the potentials for underwater photosynthesis in aquatic systems considerably. Under water photosynthesis will be limited even more by the severely hampered gas exchange under water, since it reduces not only the oxygen availability, but also the inorganic carbon supply for photosynthesis (Bowes 1987, Madsen and Sand-Jensen 1994). Furthermore, the relatively high viscosity of water compared to air leads to the development of larger stagnant boundary layers around the leaves (Smith and Walker 1980, Jones et al. 2000), which even further reduce CO_2 availability for photosynthesis under water. In terrestrial plants, which are not specialized to an aquatic life, underwater photosynthesis is characterized by relatively low maximum photosynthesis rates and low uptake efficiency of CO_2 due to high diffusion resistance (Maberly and Madsen 1998, Sand-Jensen and Frost-Christensen 1999). Another result of low gas diffusion rates in these species is that the internal CO_2 concentration is relatively low compared to the internal O_2 concentration, which favors oxygenation reactions of Rubisco over carboxylation. Underwater photosynthesis is, therefore, predicted to be characterized by high photorespiration rates (Lloyd et al. 1977, Salvucci and Bowes 1982, Maberly and Spence 1989).

Despite all these reasons to expect a minor role of underwater photosynthesis for terrestrial plants, the effects of light availability under water on plant survival appear to be surprisingly large. The few studies focusing on this topic show that in the presence of light, underwater plant survival is considerably higher, the decrease in biomass is lower, and carbohydrate reserves are depleted slower than in darkness (Setter et al. 1987, Laan and Blom 1990, Nabben et al. 1999). Vervuren et al. (2003) showed that even a very limited amount of light (4-20 µmol m^{-2} s^{-1}) was sufficient for a considerable increase in underwater

survival time in several terrestrial species. In addition, the benefits of light under water increased with increasing CO_2 availability under water (Laan and Blom 1990). Thus, current evidence suggests that the availability of light is an important factor for plant survival and performance of terrestrial plants during submergence. This implies an important role for photosynthesis under water, despite of the unfavourable conditions.

Acclimation to submergence in relation to gas exchange

The degree to which plants can benefit from underwater photosynthesis will largely depend on the gas exchange capacity of their leaves under water. Earlier attempts to relate underwater photosynthesis to flooding tolerance failed to find such a correlation, as non-acclimated plants were investigated at high CO_2 concentrations (Voesenek et al. 1993b, He et al. 1999, Vervuren et al. 1999). In such non-acclimated plants, internal oxygen pressures have been shown to decrease to very low levels within 30 minutes upon submergence (Raskin and Kende 1983, Stünzi and Kende 1989, Rijnders et al. 2000). This suggests that the gas exchange capacity of terrestrial plants is insufficient for photosynthesis under aquatic conditions. However, terrestrial plants do have an increased survival of flooding in the availability of light (Nabben et al. 1999, Vervuren et al. 2003), showing that photosynthesis, and thus considerable gas exchange, does take place. This apparent contradiction might be explained by an adjustment of gas exchange capacity during acclimation to submergence. This thesis, therefore, aims to investigate in depth the effects of acclimation to submergence on underwater gas exchange capacity of terrestrial plants.

Enhancement of carbon supply under water in aquatic species

As CO_2 supply is severely limited under water, many true aquatic plant species possess CO_2 concentrating mechanisms which enhance carbon gain under water (Maberly and Madsen 2002). The most widespread mechanism to increase carbon dioxide availability is the ability to use HCO_3^- in photosynthesis (Allen and Spence 1981, Prins and Elzenga 1989, Madsen 1993). This may be achieved by proton extrusion at one side of the leaf, thereby lowering the pH and thus shifting the inorganic carbon equilibrium in favour of carbon dioxide over HCO_3^- (Prins et al. 1982, Lara et al. 2002). The conversion may be catalysed by the apoplastic enzyme carbonic anhydrase (Newman and Raven 1999). HCO_3^- itself may also be actively taken up (Elzenga and Prins 1989, Lara et al. 2002). The latter process also requires proton extrusion as a driving force, which makes distinction between the two processes difficult. The use of bicarbonate is a carbon concentrating mechanism, and often coupled to a C_4 metabolism, as has been reported for *Hydrilla verticillata* (Holaday and Bowes 1980, Spencer et al. 1996, Reiskind et al. 1997, Magnin et al. 1997), *Elodea canadensis* (Elzenga and Prins

1989) and *Egeria densa* (Browse et al. 1979, Casati et al. 2000) and references in Bowes et al. (2002) The initial carboxylating enzyme in C_4 photosynthesis is phosphoenolpyruvate carboxylase (PEPcarboxylase), which fixes carbon dioxide in the mesophyll cells into malate. PEPcarboxylase scavenges carbon dioxide more efficiently than Rubisco and is not negatively affected by high oxygen concentration, like Rubisco is. The formed malate then diffuses to the bundle sheath cells, where decarboxylation releases carbon dioxide, which is subsequently fixed by Rubisco. This type of metabolism generally requests a spatial separation between the C_3 and C_4 carboxylating enzymes, but the characteristic Kranz or bundle sheath anatomy observed in terrestrial plants (Lambers et al. 1998) is most often lacking in aquatic species (Reiskind et al. 1997, Magnin et al. 1997). Separation between the C_3 and C_4 carboxylating enzymes in aquatic species, however, appears to occur at the cellular level at the chloroplasts (Reiskind et al. 1997, Casati et al. 2000, Rao et al. 2002).

Several true aquatic plants show an alternative carboxylation pathway, crassulacean acid metabolism (CAM), which is also observed in drought-adapted plant species. This alternative carboxylation pathway has a separation of the two carboxylation steps in time. Uptake of carbon dioxide and subsequent assimilation into malate takes place during night during which malate accumulates in the tissues, whereas the secondary carboxylation by Rubisco takes place during daytime. CAM has only been observed in isoetids, such as *Lobelia dortmanna* and *Littorella uniflora* (Robe and Griffiths 1990, Madsen et al. 2002). These species can use carbon dioxide from the sediment, which contains high carbon dioxide concentrations as a result of microbial respiration, as additional carbon supply (Wium-Andersen 1971, Roelofs et al. 1984, Pedersen et al. 1995). This carbon dioxide diffuses from the soil into the roots and then further follows the concentration gradient into the shoot via aerenchymatous tissue.

In both C_4 and CAM photosynthesis, CO_2 compensation points are lower and photorespiration rates are strongly suppressed compared to C_3 photosynthesis. The occurrence of photorespiration under water has been demonstrated extensively in the past by measuring CO_2 compensation points in aquatic plants in water-saturated air at low and high O_2 concentrations (Van et al. 1976, Lloyd et al. 1977, Salvucci and Bowes 1983). These indirect measurements were often supported with enzyme assays and labeling studies of the photorespiratory cycle and C_4 metabolism (Salvucci and Bowes 1982, Salvucci and Bowes 1983, Spencer et al. 1996) but accurate *in vivo* measurements of underwater photorespiration rates are lacking up till now.

Another strategy of plants growing in aquatic systems to enhance carbon gain is the development of aerial leaves next to their aquatic counterparts (Sand-Jensen and Frost-Christensen 1999, Kuwabara et al. 2001), which provides direct access to gaseous CO_2 in air. These species show remarkable plasticity in leaf form, specialized for either

photosynthesis in air or under water (Maberly and Spence 1989, Wells and Pigliucci 2000). This shows that plasticity can exist for gas exchange capacity upon submergence, and this thesis will explore if terrestrial plants can employ this strategy.

Enhancement of under water carbon uptake in terrestrial and amphibious species

In contrast to terrestrial plants, morphology and underwater photosynthesis of terrestrial and aquatic leaf types of amphibious plants have been studied extensively (Maberly and Spence 1989, Nielsen and Sand-Jensen 1989, Sand-Jensen and Frost-Christensen 1999, Germ and Gaberscik 2003). Amphibious plants have, in extreme cases, a specialized aquatic leaf form with filamentous, dissected leaves and no or only few stomata, which is entirely different from the terrestrial form (Bruni et al. 1996). Most aquatic leaves of amphibious plants, however, are elongated and thinner and with a higher specific leaf area (SLA) than the terrestrial leaves (Nielsen 1993, Frost-Christensen and Sand-Jensen 1995). Sand-Jensen et al. (1992) and Frost-Christensen and Sand-Jensen (1995) showed that aquatic leaves of these species have increased underwater photosynthesis rates and a higher CO_2 affinity compared to their terrestrial counterparts. This may be the result of reduced gas diffusion resistance of the aquatic compared to the terrestrial leaves. Frost-Christensen et al. (2003) showed that the reduced gas diffusion resistance in aquatic leaves of amphibious plant species originated from reduced cuticle thickness and its reduced diffusion resistance for gasses such as O_2. The acclimation of aquatic leaves is therefore considered to be predominantly directed to optimization of gas exchange of these leaves (Madsen and Maberly 1991, Nielsen 1993). However, there are suggestions that the differences between leaf types should be interpreted as a constitutive shade morphology of aquatic leaves (Bowes and Salvucci 1989, Boeger and Poulson 2003). Next to differences in diffusion resistance, also differences at the biochemical level of photosynthesis have been observed between the leaf types of amphibious plants. Chlorophyll contents (Frost-Christensen and Sand-Jensen 1992, Nielsen 1993), as well as concentrations of the carboxylation enzymes Rubisco and PEP carboxylase (Farmer et al. 1986, Beer et al. 1991) are lower in aquatic compared to terrestrial leaves. This probably results from the reduced leaf thickness of the aquatic compared to terrestrial leaves of amphibious plants, and may lead to reduced light absorption and carboxylation capacity.

Rumex palustris as a model

Several species from the genus *Rumex* are distributed over the flooding gradient, ranging from relatively intolerant species such as *R. acetosa* and *R. thyrsiflorus* at rarely flooded sites at relatively high elevation to flooding resistant species such as *R. maritimus* and *R. palustris*

growing on frequently flooded mud flats (Blom et al. 1994). The different species within this genus show different levels of flooding resistance which corresponds with variation in life history (Van der Sman et al. 1993) and physiological characteristics (Blom et al. 1994, Visser et al. 1996a). In the recent years, the tolerant floodplain species *Rumex palustris* has become a model species for plant responses to submergence (Visser et al. 1996b, Cox et al. 2003, Voesenek et al. 2003b). This species develops aerenchymatous adventitious roots upon partial soil flooding, and enhanced petiole elongation during full submergence in order to restore contact with the atmosphere. This species also shows a high plasticity in leaf morphology upon submergence, which makes it a suitable system for studying phenotypic plasticity in plants. The submerged leaf morphology might facilitate gas exchange under water, and have implications for underwater photosynthesis. *R. palustris* is, therefore, used in this thesis as a model species.

Outline of thesis

This thesis will investigate the effects of acclimation to submergence in relation to under water gas exchange and photosynthesis. The role of light for plant survival under water will be investigated in a multi-species approach in chapter 2. It shows the relationships between a set of submergence-related ecophysiological traits and their effects on plant survival during submergence in order to improve our understanding of the traits determining flooding tolerance. Chapter 3 aims to elucidate to what extent submergence and shade induce similar acclimation responses by comparing two terrestrial *Rumex* species that differ in their response to flooding. These broad comparative studies will be followed by in-depth investigations of the parameters that determine the enhanced gas exchange capacity that accompanies acclimation to submergence. In chapter 4 the effects of acclimation to gas exchange under water were investigated. Using oxygen microelectrodes, the oxygen dynamics were recorded in dark and light in submergence-acclimated and non-acclimated leaves of *Rumex palustris*. The photosynthetic consequences of the increased gas exchange capacity are further evaluated in chapter 5. To this purpose, the anatomy of submergence-acclimated and non-acclimated leaves of *Rumex palustris* was studied, as well as underwater and aerial photosynthesis rates in both leaf types. The combination of gas exchange and chlorophyll fluorescence measurements enabled *in vivo* estimates of photorespiration under water. Finally all results are briefly summarized and discussed in chapter 6, allowing a quantification of the effect of acclimation to submergence for gas exchange.

Chapter 2

Ecophysiological determinants of plant performance during submergence: a comparative study among seven plant families

with: John P. M. Lenssen, Heidrun Huber, Eric J.W. Visser and Hans de Kroon

Abstract

Plant growth and performance of species in river floodplains is negatively affected by submergence. Several individual traits, e.g. elongation capacity and aerenchyma content have shown to determine flooding tolerance, but the interrelationships among these traits and their effects on plant performance still remain largely unknown. Here, we aim for a more coherent understanding of submergence tolerance, by investigating whether different suites of traits are alternative strategies to enhance survival or if they act in concert. Since responses to submergence may be taxon-specific, we chose a multiple-species approach that allowed phylogenetic comparisons. The availability of light during flooding was expected to modify plant performance and trait expression, and therefore, all responses were measured in dark and light.

The traits investigated in the current study, plant height, aerenchyma content of the petiole, specific leaf area (SLA), leaf longevity and chlorophyll content were constitutively higher in species originating from frequently flooded habitats than in species from dry habitats. Moreover, these 'wet' species appeared to be more plastic for these traits in response to submergence. Neither the phylogenetic component nor the light climate under water were important for the expression of the submergence-related traits.

Principal component analysis (PCA) showed that these traits were divided in two independent clusters structured either around the leaf lamina (i.e. SLA, chlorophyll and leaf longevity) or the petiole (i.e. aerenchyma content and plant height). Interestingly, a positive rather than negative correlation between SLA and leaf longevity was observed under water, indicating an increased potential for underwater photosynthesis through enhanced gas exchange in thinner leaves. Plant height and aerenchyma content of the petiole appeared also positively correlated. Path analyses indicated significant positive effects of these two clusters of traits on plant performance during full submergence. The broad palette of traits

acting in concert may fine tune the responses to flooding, which is a prerequisite in such unpredictable and dynamic environments such as river floodplains.

Introduction

Plant species from river floodplains experience flooding frequently but at irregular time intervals (Vervuren et al. 2003). The huge impact of submergence on plant performance is reflected in sharp lower distribution limits of plant species along natural flooding gradients (Menges and Waller 1983, Sýkora et al. 1988, van Eck et al. 2004). The differences among species in their lowest position on such gradients indicate that plant species differ in their ability to cope with flooding (Blom et al. 1994, Blom 1999). These differences in flooding tolerance among species may be caused by differential expression of traits that reduce the negative effects of submergence (Armstrong et al. 1994a).

The reduced plant performance under submergence is the consequence of hampered gas exchange between the plant and the environment due to the low diffusion rate of gases in water. Reduced gas exchange results in oxygen deficiency and energy shortage (Blom and Voesenek 1996, Vartapetian and Jackson 1997). The availability of light under water might alleviate the negative effects of submergence since in the presence of light oxygen and carbohydrates can be produced during photosynthesis. Submergence, however, may reduce light intensities due to high turbidity of the flood water (Vervuren et al. 2003), but even very low light intensities (4-20 μmol m^{-2} s^{-1}) have been shown to be sufficient to considerably increase survival of flooded terrestrial plants (Nabben et al. 1999, Vervuren et al. 2003)

Phenotypic traits that are considered to be of major importance during submergence are elongation of shoot organs to restore contact with the atmosphere (Kende et al. 1998, Voesenek et al. 2003a), gas exchange capacity between water column and the leaves (He et al. 1999, Vervuren et al. 1999) and increased amounts of air channels (aerenchyma) in shoot and roots to enhance within-plant gas diffusion (Jackson and Armstrong 1999, Colmer 2003). The adaptive value of these traits has been mainly derived from interspecific (Justin and Armstrong 1987, He et al. 1999, Nabben et al. 1999, Visser et al. 2000, McDonald et al. 2002, Voesenek et al. 2004, van Eck et al. 2005) and a few intraspecific (Chan and Burton 1992, Robe and Griffiths 1998, Lynn and Waldren 2002, Lenssen et al. 2004) comparisons of species from frequently and rarely flooded sites.

However, whether these trait variations between species from frequently and rarely flooded sites are solely due to adaptations to flooding has not been unequivocally demonstrated. First, since ecophysiological traits may be phylogenetically constrained (van Groenendael et al. 1996, Silvertown et al. 2001) trait variation (or the lack of it) may be taxon specific. Secondly, interspecific comparisons typically focus on one trait, thus ignoring

phenotypic relationships with other traits (Ackerly et al. 2000) even when they include a broader range of taxa (Justin and Armstrong 1987, Voesenek et al. 2004). As a consequence, trait variation between species from frequently and irregularly flooded sites may in fact be due to relationships with other more important traits or may only affect tolerance to flooding indirectly.

Novel insights will originate from the combination of analyzing multiple ecophysiological traits in a multiple species approach (Eviner and Chapin 2003). Here, we use such an approach to investigate which suite of ecophysiological traits determines survival under submergence. We specifically ask the question whether these different individual traits are alternative strategies to enhance survival during flooding or, on the other hand, if these traits act in concert.

We aim to elucidate the relationships between traits relevant for flooding tolerance and their effects on plant performance under flooded conditions. To this end, individual plants of 14 terrestrial species from 7 different families, from different parts of the floodplain gradient were completely submerged for 120 days in both clear and turbid floodwater. Constitutive traits as well as plasticity in these traits were monitored in combination with measures of plant performance under water.

We tested if the traits relevant for submergence have evolved only within certain taxa, indicating phylogenetic constraints, or parallel in different taxa in response to environmental conditions. The interdependence between the different inherent traits and their expression upon submergence was analyzed with principal component analysis (PCA), investigating if phenotypic traits were correlated, and thereby providing information about possible coevolution of plant responses to submergence. Next, the effects of the traits on plant performance were analyzed using selection analyses. Life history traits have been investigated in relation to submergence in *Arabidopsis thaliana* (Kolodynska and Pigliucci 2003), but ecophysiological traits have not often been incorporated in path analyses (Arntz and Delph 2001). This study is the first attempt to incorporate several ecophysiological traits in this type of studies allowing for a much more integrated understanding of submergence tolerance.

Materials and methods

Species selection and plant material

We investigated the responses of plants of 7 families each represented by one species from frequently flooded, relatively wet habitats and one species from drier, higher elevated habitats in the floodplains of the river Rhine in the Netherlands (Table 1). Although the precise elevational range differed among species, wet habitats were always within the range of 10 m (mean water level between 1950 and 1999 at Lobith, The Netherlands) and 13 m above sea level and experienced on average 20 days of flooding during the growing season

Table 1. Species pairs selected within 7 families representative for the floodplains of the river Rhine. Habitat classifications 'dry' and 'wet' refer to high and low elevated sites of the floodplain respectively, see for details, materials and methods. References give information on habitat occurrence of the species along the floodplain gradient.

Family	Habitat	Species	Abbrev.	Symbol	Reference
Apiaceae	dry	*Daucus carota* L.	Daucar	○	van Eck et al., 2004
	wet	*Oenanthe aquatica* (L.) Poir.	Oenaqu	●	Hroudova et al., 1992
Lamiaceae	dry	*Salvia pratensis* L.	Salpra	□	Sýkora et al., 1988
	wet	*Mentha aquatica* L.	Menaqu	■	Sand-Jensen et al., 1992
Rosaceae	dry	*Potentilla verna* L.	Potver	△	Poschlod and Jackel., 1993
	wet	*Potentilla reptans* L.	Potrep	▲	van Eck et al., 2004
Ranunculaceae	dry	*Ranunculus bulbosus* L.	Ranbul	◇	He et al., 1999
	wet	*Ranunculus repens* L.	Ranrep	◆	van Eck et al., 2004
Poaceae	dry	*Arrhenatherum elatius* (L.) J.&C.Presl	Arrela	⬠	van Eck et al., 2004
	wet	*Phalaris arundinacea* L.	Phaaru	⬟	Vervuren et al., 1999
Polygonaceae	dry	*Rumex thyrsiflorus* Fingerh.	Rumthy	▽	van Eck et al., 2004
	wet	*Rumex palustris* Sm.	Rumpal	▼	Voesenek et al., 2004
Asteraceae	dry	*Achillea millefolium* L.	Achmil	⊡	Vervuren et al., 2003
	wet	*Achillea ptarmica* L.	Achpta	◘	van Eck et al., 2004

(Van de Steeg and Blom 1998, Vervuren et al. 2003). Species from dry habitats were restricted to positions above 13 m with a maximum flooding period of 5 days per growing season. Seeds of the 14 species were collected from populations in the floodplains near Nijmegen (the Netherlands) and germinated for 10 days on moistened filter paper in petri dishes (temperature 22°C during daytime and 10°C at night; Photosynthetic Photon Flux Density (PPFD) was 20 µmol m^{-2} s^{-1}. To increase germination percentages of several species, gibberellic acid (0.1 mM) was sprayed into the petri dishes. The seedlings were transplanted into pots of 6x6x8 cm containing a sieved sand/clay mixture (1:1 v:v), and

grown for another 6 weeks in a growth chamber (PPFD at leaf level 250 µmol m^{-2} s^{-1} (SON-T plus 600 W and TLD Reflex 36W/840R, Philips, Eindhoven, the Netherlands); day/night cycle 16h/8h; temperature 20°C). To prevent nutrient deficiency, plants were watered once a week with ¼ Hoagland's nutrient solution, from the third week onwards. The positions of the pots were changed regularly to homogenize the growth conditions among replicate plants.

Experimental design

Plants were completely submerged in 10 opaque polyethylene basins (80x60x70 cm), which were serially connected to each other and placed in a growth chamber (SON-T plus 600 W and TLD Reflex 36W/840R, Philips, Eindhoven, the Netherlands; day/night cycle 16h/8h; temperature 20°C). The basins were filled with tap water, which was circulated with a flow rate of 1.5 L min^{-1}. To prevent algal growth the water was continuously filtered and every other week 20% of the water was refreshed. The total dissolved inorganic carbon concentration of the water in the basins, measured with an infra red gas analyzer (Horiba PIR 2000; Horiba Instruments Ltd., Northampton, UK) was 1.3 mM, which results at pH 8.4 and a temperature of 20°C in a free CO_2 concentration of 15 µM. The oxygen concentration of the water was at air-saturation. Plants were prevented from reaching the water surface by means of plasticized metal gauze a few cm below the water surface.

Plants were submerged in complete darkness or in light conditions to simulate turbid and clear floodwater respectively. Five basins assigned to the dark treatment (0 µmol m^{-2} s^{-1}) were covered with black sheets. Each of the five basins assigned to the light treatment was covered with neutral filters (type ULS 10, Ludvig Svensson, Hellevoetsluis, the Netherlands) to attain a light intensity of 30 µmol m^{-2} s^{-1} at leaf level, which closely resembled light conditions of the Rhine river system during flooding (Vervuren et al. 2003). Fitness derivatives and morphological and physiological plant traits were recorded at several points in time on different sets of replicate plants (see Table 2), to a maximum of 120 days. Because of space limitation only the two species of a single family could be investigated simultaneously. Since all experiments were performed in the same strictly controlled growth chamber we assumed potential family effects not to be related to the different periods in which the families were tested. All experiments were performed in the period from December 2000 until December 2002.

Measurements

As parameters for plant performance in response to light and dark submergence we determined survival and relative growth rate, although for most species-treatment combinations this last parameter should be interpreted as relative decay rate, since there was a net decrease of biomass (van Eck et al. 2004). We used both survival and biomass

Table 2. Plant performance and ecophysiological traits measured in the experiment. The 'set' column indicates which parameters are obtained from the same sets of plants. The start of the submergence treatment in light and dark is indicated with t=0.

Parameter	time scale (d)	# replicates	Set
Plant performance			
Survival of plants (LT50)	0 - 120 (1)	8 x 10 plants	1
Relative growth rate	0-11,0-42	3 x 10 plants	2
Ecophysiological traits			
Plant height and elongation rate	0,11,42	3 x 10 plants	2
Specific leaf area of plant	0,11,42	3 x 10 plants	2
Aerenchyma content of petioles	0,11	2 x 6-10 plants	3
Chlorophyll content of leaves	0,11	2x 6-10 plants	3
Longevity terrestrial leaves	0 – 120 (2)	10 plants	4
Longevity aquatic leaves	14 - 120 (2)	10 plants	4

(1) Harvest dates for determination of plant survival depended on expected survival time and light intensity underwater. Guidelines for these harvests were for species from dry habitat in dark: 3,4,11,16, 20,25, 30, 35 days after submergence; dry habitat light: 5,11, 16, 23, 30, 36, 42, 50 days after submergence; wet habitat dark: 11, 20, 28, 35, 42, 49, 56, 65 days after submergence; wet habitat light: 11, 35, 50, 65, 75, 90, 120 days after submergence.

(2) Longevity of leaves was recorded every 14 days until all leaves of a given cohort had died.

loss as fitness derivatives, because they behave differently during flooding (van Eck et al. 2004). To determine survival under water we removed ten replicates per species-treatment combination from the basins at 8 dates with intervals depending on earlier investigations (Table 2; Vervuren et al. 2003, van Eck et al. 2004) After a de-submergence period of ten days, plants were considered to have survived if green, turgid leaves and/or buds were present. Relative growth rates (RGR) were recorded from total dry weight of plants harvested at the start of the experiment and after 11 and 42 days of submergence in dark and light. Total dry weight was determined after drying for at least 48 h at 80 °C.

Several ecophysiological traits considered to be of importance in flooding tolerance were investigated on different sets of plants (Table 2). These phenotypic traits were measured at the start of the treatment and after 11 and 42 days of submergence, but because of mortality of the plants of several species measurements at time point 42 were not included in the analyses. The plant response to restore contact with the air is elongation of the shoot (Voesenek et al. 2004), which includes re-orientation of the leaves (hyponasty; Cox et al. 2003) and elongation of the petiole and lamina (Voesenek et al. 2003). We recorded these traits as an integral in terms of plant height. Elongation rate was expressed as relative elongation rate by dividing the difference in plant height (ln transformed) at two harvests by

the time difference between those harvests. Specific Leaf Area (SLA, m^2 kg^{-1}DW) is in aquatic plant literature commonly used as a measure of leaf thickness (Goldsborough and Kemp 1988, Nielsen 1993), which is relevant under water since it might correlate with gas exchange capacity between the plant and the water layer. Area of the lamina was measured with a leaf area meter (LI 3000, Li-Cor, Lincoln, Nebraska, USA) and the shoot dry weight was determined after drying for at least 48 h at 80 °C. Transport capacity for gasses from the leaves into the roots determines oxygen status in the roots (Colmer 2003) and can be reflected by the amount of air channels (aerenchyma) within the petiole, using the microbalance method as described in Visser and Bögemann (2003). Vervuren et al. (1999) showed that a collapse of the chlorophyll content in three terrestrial species was proportional to the decrease in under water photosynthetic performance. To obtain an indication about the change of the photosynthetic capacity under water, chlorophyll content of the youngest full-grown leaves was determined spectrophotometrically (Shimadzu Benelux, Den Bosch, the Netherlands) after extraction with 96% ethanol. Equations of Wintermans and de Mots (1965) were used to calculate the chlorophyll a and b pigment concentrations.

Leaf longevity was recorded as a trait which reflects leaf senescence under water (Banga et al. 1997). Some species show a rapid turnover of the photosynthetic active area by continuous production of new leaves, whereas others defoliate completely or do not show any change in leaf production (De Simone et al. 2002). Leaf longevity of individual terrestrial leaves, present at the start of submergence, and of aquatic leaves, i.e. those that had emerged within the first 14 days of the experiment was recorded at regular intervals of 14 days from the start of submergence with a maximum of 120 days.

Data analysis

In all analyses that addressed the relative contribution of treatment, family and habitat to variation in fitness and plant traits we considered light treatment as a fixed factor and family as a random factor. Because the exact elevational range of wet and dry species differed among families we considered habitat as a family specific trait and treated it therefore as a fixed effect nested within family.

Survival time of species and leaf longevity of terrestrial and aquatic leaves were expressed as median lethal time (LT50), estimated by fitting survival as a function of flooding duration with a Weibull model (Egli and Schmid 2001, Vervuren et al. 2003). Plant survival and leaf longevity were analyzed with the SAS procedure LIFEREG (SAS 8.2 2001) with the Weibull model as the baseline function (Vervuren et al. 2003) and family, habitat(family), light and interactions as covariates. We then calculated ratios of mean deviance changes, which approximately follow the F-distribution (McCullagh and Nelder 1991) and will hereafter be

referred to as Quasi F-values. Mean deviances allowed us to test differences among factors against the appropriate error terms.

RGR was calculated following the classical approach as described in Poorter (1989) and pairing of the plants at different harvests was done at random, which might have overestimated the variance. Using a similar model as in the analysis of deviance we also tested relative growth rates (RGR) with ANOVA in PROC GLM.

Ecophysiological traits were also analyzed with ANOVA using PROC GLM, but in this case also Time (at start and 11 days after submergence) was added as a fixed factor to the model. The design was not always balanced, due to mortality of plants. Only samples that consisted of at least 3 replicates were included in the analyses. To achieve homogeneity and normal distribution of variances the data of plant height, specific leaf area, and chlorophyll content were square root transformed and porosity data were arc sin square root transformed prior to analysis.

Principal component analysis (PCA)(SPSS 12.0) was used to identify the internal correlation structure among the 11 different plant traits of the 14 species in order to identify the traits which were most responsive to submergence. Separate PCA analyses were performed for submergence in light and dark to investigate if the internal correlation structure between plant traits depended on light conditions under water. Prior to this analysis all variables were standardized to zero mean and unit variance. The first and second PCA axes were regressed as independent variables on survival data in linear regression analysis (SAS 8.2, PROC GLM). Species survival data were log transformed (logLT50) to improve normality. RGR was not included in the analysis, since this fitness derivative had only limited power to discriminate between species as most of the sensitive species quickly lost all biomass.

Path analysis, a statistical technique to test a priori defined direct and indirect relations between ecophysiological traits on plant species survival under flooding (logLT50), was performed with the program package AMOS (Arbuckle and Wothke 1999). Since we expected traits after acclimation to submergence to be of more importance for plant performance than constitutive trait values we included plant height, aerenchyma content, and SLA after 11 days of submergence in the model. Longevity of terrestrial rather than aquatic leaves was used in the analysis, because several species originating from dry habitats did hardly develop any aquatic leaves in dark. We calculated direct effects of SLA, plant height, aerenchyma content of the petiole and leaf longevity on species survival, taking into account correlations between these traits. An indirect direct effect of SLA through leaf longevity on survival was included since SLA is well-known to affect leaf longevity (Reich et al. 1999).

Results

Effects of habitat of origin, family and light on plant performance

Species originating from wet habitats survived significantly longer and lost less biomass than species from dry habitats (Fig 1,2; Table 3). On a longer time scale, i.e. 42 days after the start of submergence, most dry species had disappeared completely. The species originating from wet habitats, on the contrary, were still present, and some of them even showed a moderate biomass increase in the light (Fig. 2b). There was no evidence for a phylogenetic component for flooding tolerance as neither the family main effect, nor the light*family interaction terms were significant. The availability of light under water increased plant survival (LT50) of all plant species under water considerably (Fig. 1, Table 3). However, the main light effect was not significant, due to the high Habitat(Family)*Light interaction (Quasi F-value=47.92, $p<0.001$), indicating that the beneficial effect of light on survival was significantly larger for wet than for dry species (Fig. 1). The beneficial effect of light on RGR was less pronounced than the effect of light on plant survival, since the wet species had generally lost little biomass in both light and dark after 11 days of submergence (Fig. 2a). After 42 days of submergence, some wet species even had a positive carbon balance in the light, whereas most species from dry habitats had died already (Fig 2b).

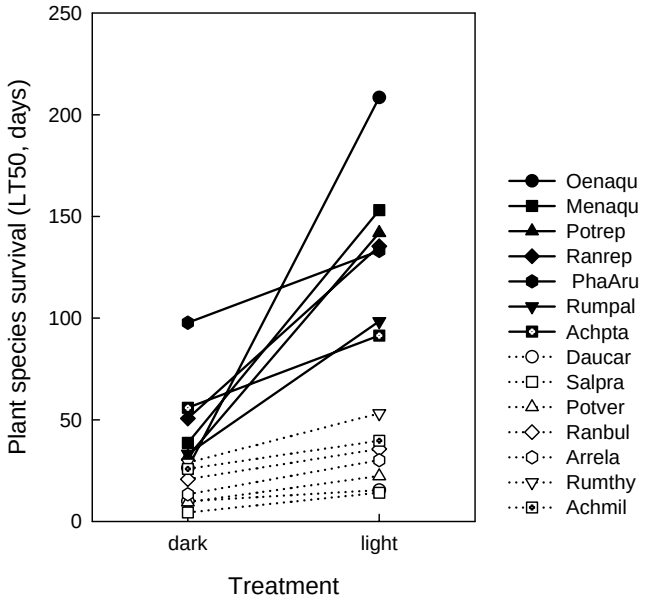

Figure 1. Survival time of 14 species originating from wet (closed symbols, solid lines) and dry habitats (open symbols, dotted lines) of the floodplain during complete submergence in dark and light conditions. Habitat means (± SE) in dark and light conditions are represented at the outer left and right of the panel.

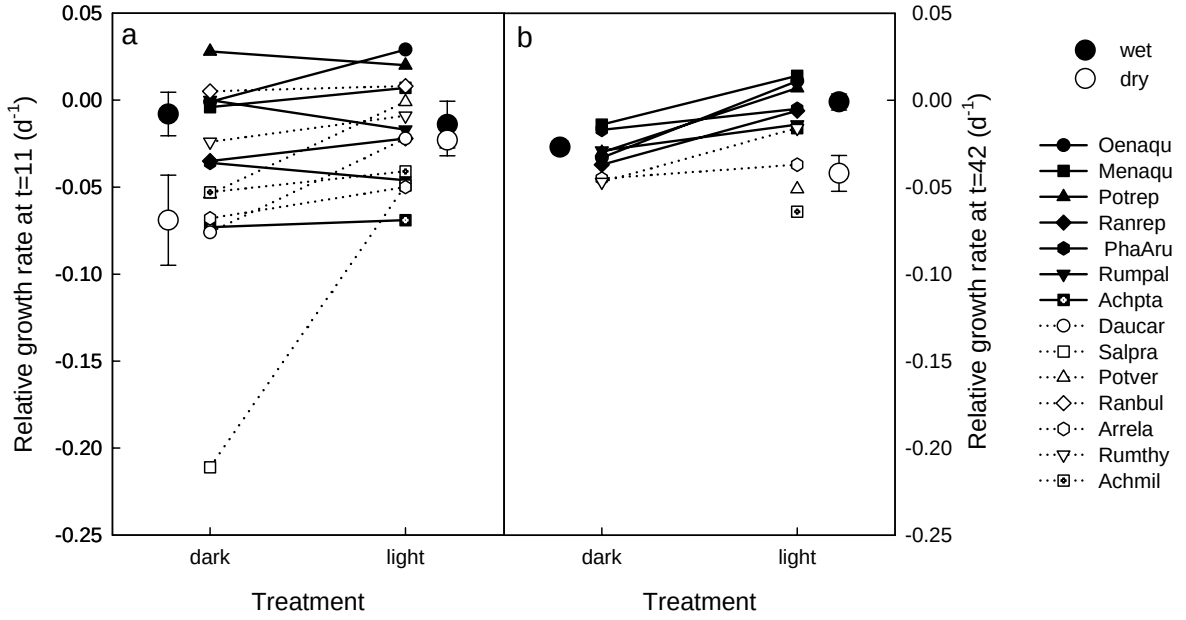

Figure 2a,b. Relative growth rate of 14 species originating from wet and dry habitats of the floodplain in dark and light conditions after (a) 11 and (b) 42 days of submergence. Species symbols as in Fig. 1. Habitat means (± SE) in dark and light conditions are represented at the outer left and right of the panels.

Effects of habitat of origin, family and light on ecophysiological traits

Most ecophysiological traits – plant height, specific leaf area (SLA) and chlorophyll content, but not aerenchyma content - changed significantly upon submergence (Fig. 3a-d, Table 4 significant Time effect). Habitat of origin had a significant main effect on these phenotypic traits (Fig. 3) as well as on leaf longevity under water (Fig. 4; Table 3, 4 significant Habitat(Family) effect), indicating inherent differences in these traits which are considered to be of importance for flooding tolerance. Moreover, depending on habitat of origin, plant height, SLA, chlorophyll content and leaf longevity significantly differed in their response to submergence in general (Table 3,4 significant Time*Habitat(Family)) and in their response to light under water (Table 3,4 significant Time*Light* Habitat(Family)). The phylogenetic component was unimportant for traits related to submergence tolerance, since only chlorophyll content showed a slight main family effect (Table 4).

Longevity of both terrestrial and aquatic leaves increased significantly when submerged in light compared to darkness (Fig. 4a,b; Table 3 significant Light effect), while chlorophyll concentration was significantly lower in darkness than in light. For the other ecophysiological traits, however, the submergence induced responses were not affected by light availability (Fig. 4a-d, Table 4 Time*Light n.s.).

Table 3. Effects of light treatment, family and habitat and their interactions on F-values of survival time of submerged plants, relative growth rate (RGR) after 11 days submergence and longevity of leaves present at start of submergence ('Ter. Leaves') and new leaves developed during the first 14 days of submergence ('Aq. Leaves'). Survival times of plants and leaves were tested with analysis of deviance and ratios refer to Quasi F-values. Relative growth rate was tested with analysis of variance. Error term for each term is indicated. * P<0.05, **P<0.01, ***P<0.001, n.s. not significant, Residual MD/S = Mean deviance (in case of Quasi F values) or Mean sum of squares of residuals and d.f. = degrees of freedom.

				Species survival	**RGR at t=11**	**Longevity Ter. leaves**	**Longevity Aq. leaves**
term	*Source of variation*	*Error term*	*d.f.*	*Quasi F value*	*F value*	*Quasi F value*	*Quasi F value*
1	Light	4	1	0.00 n.s.	3.76 n.s.	27.07 **	16.37 **
2	Family	3	6	0.17 n.s.	0.77 n.s.	0.06 n.s.	0.17 n.s.
3	Habitat(Family)	6	7	391.8 ***	12.39 ***	677.61 ***	261.63 ***
4	Light*Family	5	6	0.07 n.s.	0.87 n.s.	0.20 n.s.	1.91 n.s.
5	Light*Habitat(Fam)	6	7	47.92 ***	3.69 ***	33.18 ***	11.62 ***
6	Resid. MD/S(d.f.)			0.56 (2098)	0.002 (247)	0.70 (15210)	0.49 (1500)

Relationships among ecophysiological traits

The first two axes of the Principal Component Analysis (PCA) explained 56 % of the variation between the ecophysiological traits in both light treatments. In light, leaf traits were clustered along the first axis and plant height and aerenchyma traits along the second axis (Fig. 5b). Specific leaf area was clustered with longevity of terrestrial and aquatic leaves, since their arrows on the x-axis pointed in the same direction (Fig. 5b). Specific leaf area (SLA) was

Table 4. Effects of time, light treatment, family (Fam) and habitat and their interactions on F-values of plant height, specific leaf area (SLA), aerenchyma content of petioles and chlorophyll content per leaf area. All traits were tested with analysis of variance and error term for each term is indicated in 'error' column. * P<0.05, **P<0.01, ***P<0.001, n.s. not significant, Residual MS = Mean sum of squares of residuals and d.f. = degrees of freedom.

				Plant height	**SLA**	**Aerenchyma**	**Chl**
term	*source of variation*	*Error term*	*d.f.*	*F value*	*F value*	*F value*	*F value*
1	Time	6	1	28.46 **	66.82 **	1.22 n.s.	22.93 **
2	Light	8	1	7.96 *	1.1 n.s.	0.51 n.s.	22.85 **
3	Family	4	6	2.33 n.s.	1.36 n.s.	1.29 n.s.	5.85 *
4	Habitat(Family)	12	7	139.00 ***	94.89 ***	75.57 ***	16.44 ***
5	Time*Light	10	1	4.53 n.s.	1.87 n.s.	0.39 n.s.	26.01 **
6	Time*Family	7	6	2.73 n.s.	0.7 n.s.	1.53 n.s.	0.83 n.s.
7	Time*Habitat(Fam)	12	7	36.74 ***	14.57 ***	9.07 ***	23.45 ***
8	Light*Family	9	6	1.03 n.s.	2.27 n.s.	0.43 n.s.	3.08 n.s.
9	Light*Habitat(Fam)	12	7	4.16 ***	5.81 ***	1.23 n.s.	2.63 *
10	Time*Light*Family	11	6	1.06 n.s.	2.47 n.s.	0.30 n.s.	2.31 n.s.
11	Time*Light*Hab(Fam)	12	7	5.59 ***	2.55 *	1.63 n.s.	2.9 **
12	Residual MS (d.f.)			0.14(487)	1.67(351)	0.004 (281)	2.63(224)

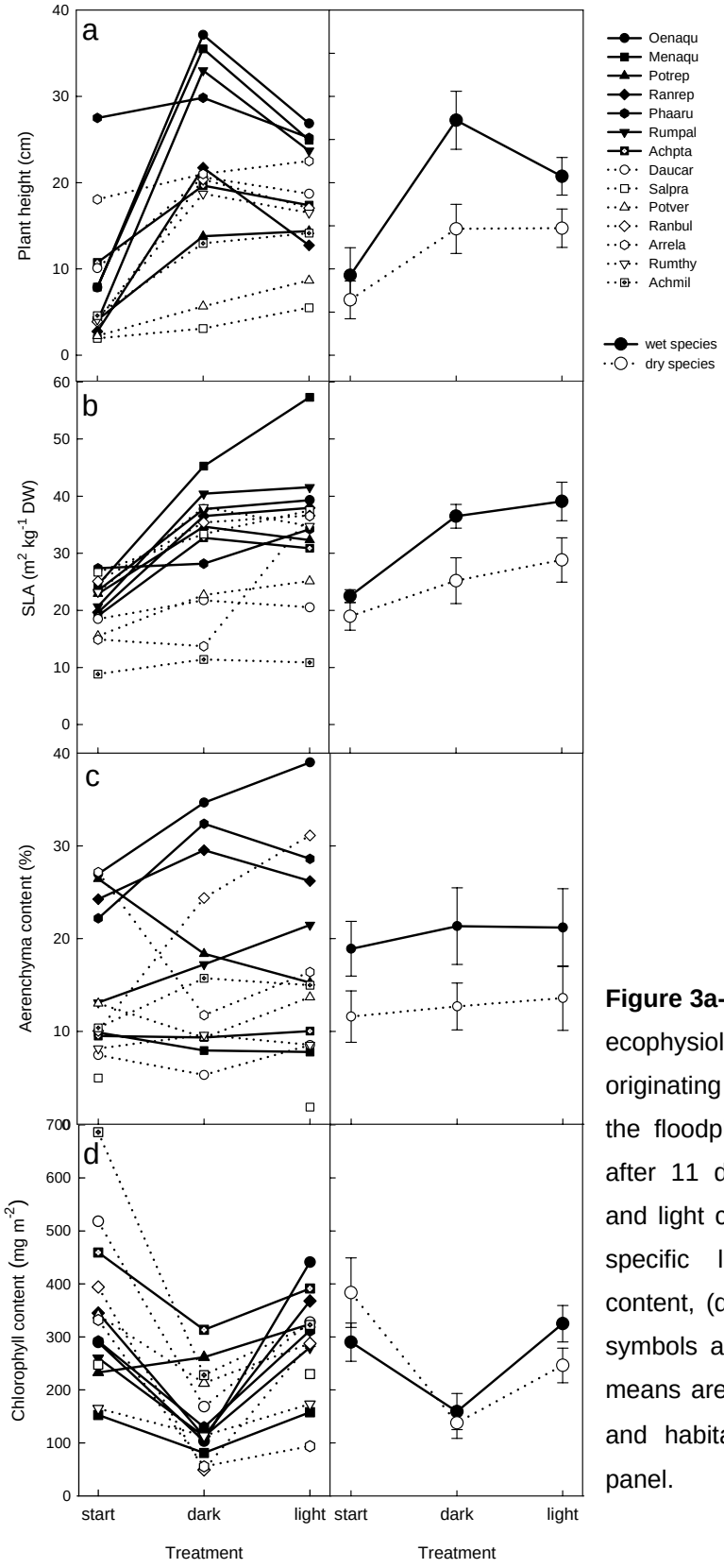

Figure 3a-d. Parameter values of several ecophysiological traits of 14 species originating from wet and dry habitats of the floodplain at start of treatment and after 11 days of submergence in dark and light conditions: (a) plant height, (b) specific leaf area, (c) aerenchyma content, (d) chlorophyll content. Species symbols as in Fig. 1. Separate species means are represented in the left panel, and habitat means ± SE in the right panel.

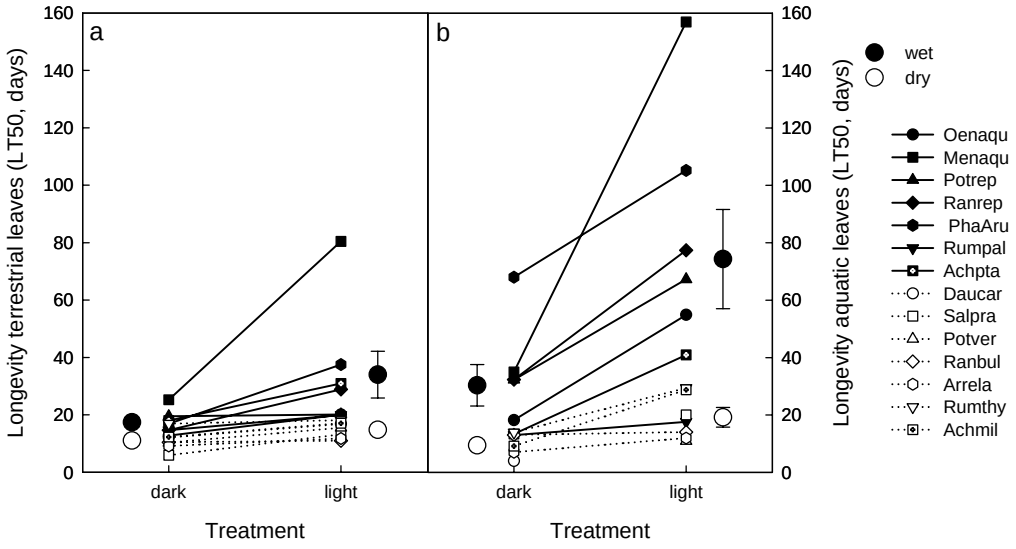

Figure 4a,b. Longevity of (a) terrestrial and (b) aquatic leaves of 14 species originating from wet and dry habitats of the floodplain during submergence in dark and light conditions. Species symbols as in Fig. 1. Habitat means (± SE) in dark and light conditions are represented at the outer left and right of the panel.

negatively correlated with chlorophyll content, especially at the start of the experiment, as demonstrated by their exact opposite scores on the x-axis. Aerenchyma and plant height parameters varied independently from leaf characteristics, since their lines in the PCA plot are positioned perpendicular to each other (Fig. 5b) and thus showed a strong correlation with the second PCA axis.

Furthermore, relative elongation rate was negatively correlated to plant height (Fig. 5a,b), which might be the result of an ontogenetic constraint. Plant species that were inherently faster increase in height were already closer to their maximum height at the start of the submergence treatment than smaller species, resulting in lower elongation rates for the taller species.

The general pattern observed in the light was also visible in the dark, although there were a few differences (Fig. 5a). In dark the parameters plant height and aerenchyma content after 11 days of submergence were less independent from leaf traits than in light. Furthermore, the correlation between longevities of aquatic leaves and terrestrial leaves was weaker than observed in light conditions.

Table 5. Standardised regression coefficients (B) and their significance (P) of PCA scores in dark and light. Survival time is expressed as the logarithm of median survival time (logLT50). * P<0.05, n.s. not significant. n=14.

	logLT50 dark		**logLT50 light**	
	B	P	B	P
PCA(1)	0.191	*	0.257	*
PCA(2)	0.091	n.s.	0.080	n.s.

Relationships between plant performance and ecophysiological traits

Univariate regressions of the first PCA axis on plant survival were significant in dark and light conditions (Table 5), suggesting a strong effect of leaf characteristics on fitness of the flooded plants. In contrast, the second PCA axis was not significantly correlated to plant performance in either light or dark.

The path analyses performed to investigate the relationships between phenotypic traits in response plant survival under submergence, revealed χ^2 values of 3.3 (P=0.19) and 3.4 (P=0.19) for flooding in darkness and light respectively, indicating that the proposed

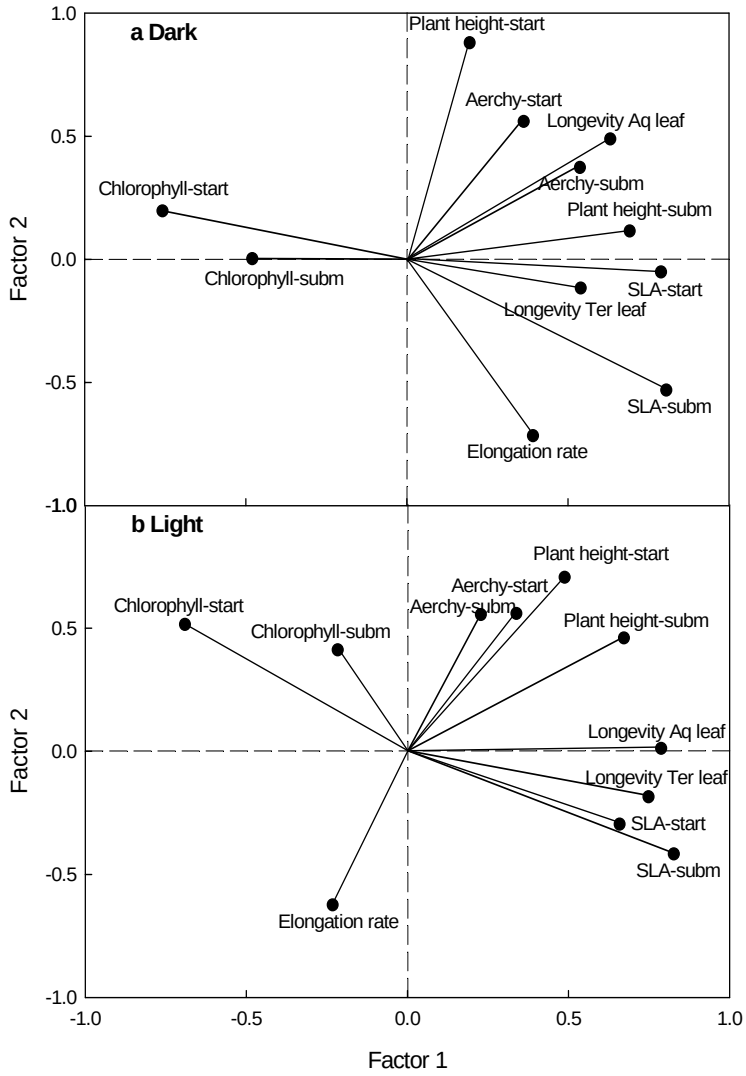

Figure 5a,b. Principal components analysis of phenotypic traits during submergence in (a) dark and (b) light conditions. In both figures, the factor loadings of the PCA-axes 1 and 2 are shown, which explain in darkness 35 and 21 % and in light 34 and 22% of the variation. Angles between vectors indicate the degree of independence of individual variables. 'SLA' means specific leaf area, 'Aerchy' aerenchyma content, 'Aq' aquatic and 'Ter' terrestrial; '-start' indicates the constitutive value of a trait as expressed at the start of the experiment, '-subm' the value after 11 days of submergence. Data are standardized to mean zero and unit deviance prior to analysis.

models adequately described the underlying relationships. Plant height and SLA did not have significant direct effects on plant survival in both light conditions (Fig. 6a,b). SLA, however, did have a significant indirect effect via leaf longevity, which in turn had a strong significant effect on fitness of plants under water. The direct effect of aerenchyma of the petioles on plant survival under water was significant, whereas correlations between SLA, plant height and aerenchyma content were not.

Again, the adaptiveness of ecophysiological traits did only slightly depend on the light conditions under water; the general pattern was similar between light and dark and only the strength of the effects differed. The signs of the direct effect of SLA, however, was opposite in the different light environments under water.

Discussion

The results of this study suggest that traits related to flooding tolerance have evolved repeatedly in response to environmental conditions across taxa of phylogenetic levels since we observed qualitatively similar responses in the 7 species pairs from completely different families. This makes our dataset suitable for testing the adaptiveness of traits under submerged conditions. Traits appeared to be evolved in two independent clusters organized around morphological structures as the leaf (i.e. specific leaf area (SLA), chlorophyll, leaf longevity) and the petiole (i.e. aerenchyma content, plant height). In contrast with the literature, SLA was positively rather than negatively related to leaf longevity, which shows a different selection pressure on SLA in flooded as compared to non-flooded individual plants of the same species. Strong correlations between aerenchyma and plant height suggest that they have coevolved in response to submergence. The expression of these traits was not affected by the light level at which the experiment was performed. The species inhabiting the lower frequently flooded parts possess a broad palette of traits acting in concert, thereby fine tuning the responses of the plant in such an unpredictable habitat.

SLA correlates positively to leaf longevity underwater

Two well-described correlations of traits with SLA were observed in our study. First, a negative relationship between SLA and chlorophyll was observed, which can easily be explained since leaves with a lower SLA, generally have thicker leaves and thus higher nitrogen and chlorophyll contents on a leaf area basis (Poorter and Remkes 1990, Reich et al. 1999). Secondly, there was a positive relationship between SLA and leaf longevity, which clearly contrasts with the negative correlations between these traits observed for thousands of plant species over a great variety of habitats (Reich et al. 1999, Wright et al. 2004). In our study this relationship was thus reversed, which suggests that the generally described

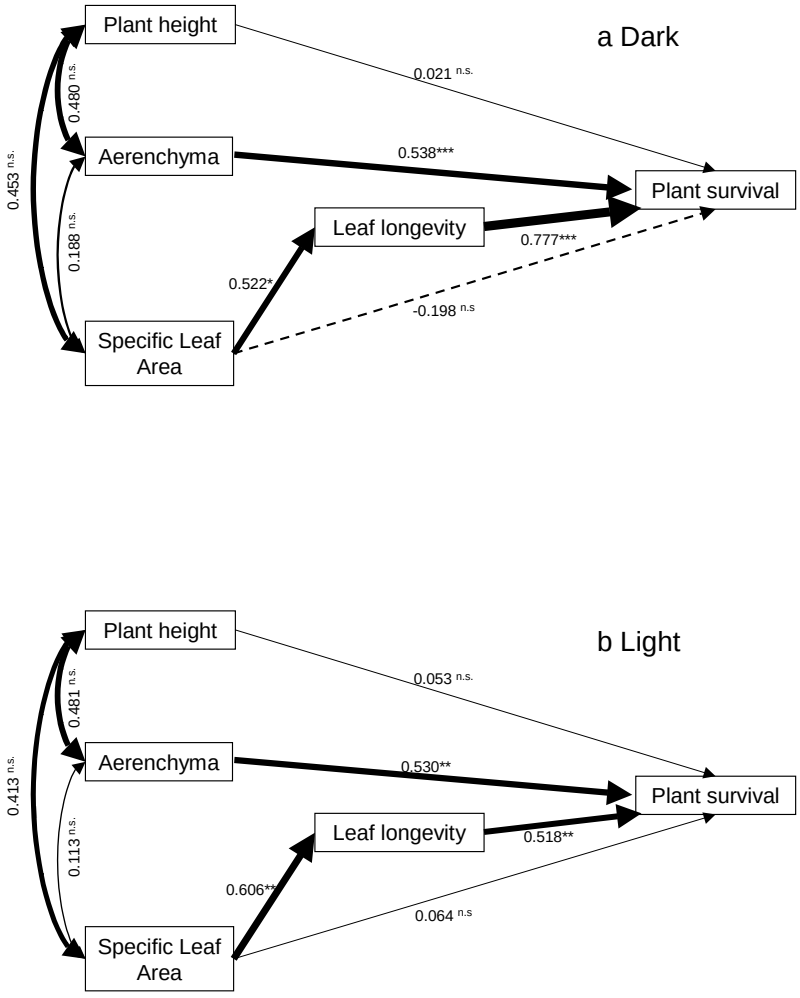

Figure 6a,b. Path diagrams showing the effects of phenotypic traits after 11 days of submergence on longevity of terrestrial leaves and plant survival during submergence in (a) dark and (b) light conditions. Standardized path coefficients are plotted, dashed lines indicate negative coefficients; the width of the arrow indicates the strength of the effect. Double arrows indicate correlations. * P<0.05, **P<0.01, ***P<0.001, n.s. not significant. Variation due to error is not included for simplicity.

negative relationship may be restricted to terrestrial plants growing in terrestrial systems and may change as soon as terrestrial plants are submerged. One explanation for this reversed relationship under water may be related to the water column that will provide some mechanical support, allowing for reduced mechanical strength as a result of reduced construction investment. More importantly, a higher SLA increases the relative surface area that is in contact with the water layer. Under water gases enter the plant via cuticle and epidermis cells as the stomata are not functioning and thus increased gas exchange area will increase gas exchange capacity. It is well known that aquatic leaves of amphibious plants have higher SLA than their aerial counterparts (Nielsen 1993, Sand-Jensen and Frost-Christensen 1998) and also that the aquatic leaves have lower gas diffusion resistance than

the aerial leaves (Frost-Christensen et al. 2003). Our results indicate that leaf longevity increases with increased SLA under water, resulting from increased potential for underwater photosynthesis through enhanced gas exchange. In air, the stomata regulate gas exchange with the environment, and SLA is not related to gas exchange, but rather to herbivory resistance or water loss. This reversed relationship in water and air may indicate contrasting selection pressures on SLA in flooded as compared to non-flooded conditions. However, plants are constraint in their response to flooding as SLA after submergence was highly correlated to SLA before submergence (in dark 56% of the variance in SLA_{11} is explained by SLA_{start}, $p<0.01$; in light 44% of the variance in SLA_{11} is explained by SLA_{start}, $p<0.01$). These correlations point to a constraint for the optimal response of an individual plant growing at the lower parts of the floodplains.

Coevolution of plant height and aerenchyma content

Longer petioles or stems have on average a higher aerenchyma content, as suggested by the second PCA axis and a relatively strong, but not yet significant correlation in the path diagram. The putative trait cluster with plant height and aerenchyma content of the petiole was unexpected. We suggest that a biophysical constraint may explain this correlation. If plants elongate upon submergence and leaves restore contact with the atmosphere, but do not develop aerenchyma channels through which atmospheric oxygen may diffuse into the roots, the roots will stay anaerobic and will eventually die. The taller a plant gets, the longer the diffusion path and the higher the within-plant diffusion resistance will be. Therefore, more aerenchyma is required to reduce this resistance for gas diffusion from the leaves to the roots (Armstrong and Beckett 1985, Armstrong et al. 1994b). The positive link between plant height and aerenchyma content therefore, suggests that these two traits coevolved in terrestrial plant species inhabiting floodplains.

Benefits from increased allocation in height growth will only arise when flooding is shallow enough to allow restoration of contact with the air (Setter and Laureles 1996, Voesenek et al. 2004). Since this was prevented in the present experiment, it is not surprising that we found no significant direct effect of plant height on plant performance under water.

Light intensity affects survival under water, but not expression of traits

The availability of only a limited amount of light enhanced plant survival under water considerably in all species (Fig. 1), confirming data of Nabben et al. (1999) and Vervuren et al. (2003). Underwater photosynthesis will have occurred in the presence of light, thereby raising the internal oxygen concentration, as shown for *Rumex palustris* plants grown under similar conditions as in this experiment (Mommer et al. 2004), and the carbohydrate status of

the plant. Gas exchange capacity is thus an important determinant for underwater plant performance, not only in light when underwater photosynthesis occurs but also in dark where passive influx of oxygen determines oxygen status of shoot and roots (Greve et al. 2003, Sorrell 2004). The species from wet habitats seem to be able to reduce gas diffusion resistance, probably by increasing SLA, thereby performing better in dark and benefiting more from the availability of light under water than do species from dry sites of the habitat.

The fact that the plants did profit substantially from the availability of only the very limited amount of light of 30 µmol photons m^{-2} s^{-1}, suggests that their light compensation point for growth may be close to or even below this value. This observation is not striking, because light compensation points observed for terrestrial herbs in sunny habitats are generally between 10 and 30 µmol m^{-2} s^{-1} (Pons 1977, Givnish 1988, Givnish et al. 2004). Not only the light compensation point of a plant species, but also the CO_2 compensation point determines the beneficial effect of light under water. The CO_2 concentration of the water was 15 µM, which is the concentration in equilibrium with air. This ambient concentration would not have resulted in net positive assimilation rates in terrestrial plants, because of the low diffusion rates of CO_2 in water and the lack of specialized CO_2 concentrating mechanisms in these plants. Still, several species, e.g. *Mentha aquatica* had a positive carbon balance at these low light intensities and CO_2 concentrations. This shows that these plants must have strongly increased their gas exchange capacity in order to enable a positive carbon balance from such a strongly limited CO_2 availability.

We expected the different beneficial effects of light on plant performance to be related to differential expression of traits under water. Surprisingly, neither PCA diagrams nor path analyses revealed large differences in expression of traits between the two light conditions under water. However, the interpretation of the PCA axes is less structured in dark than in light. Probably submergence in dark is such a severe treatment that internal correlation structures break down and responses may not be as canalized anymore resulting in more variation due to random, unmeasured effects. Alternatively, other traits may be of more importance to endure dark flooded conditions than measured in this study, such as low respiration rates (Robe and Griffiths 1998, Lenssen et al. 2004) the ability to use stored carbohydrates (Groeneveld and Voesenek 2003, Bertrand et al. 2003) and the ability to switch to an anaerobic metabolism (Perata and Alpi 1993).

Bet-hedging in response to submergence

Submerged plants may employ two strategies to overcome the negative effects of submergence: they may escape the water column or cope with the submerged conditions. Elongation of the shoot in order to reach the atmosphere is an example of the escape strategy (Voesenek et al. 2004). Selection for significant shoot elongation upon submergence

will occur on poorly dehydrating sites of the floodplain, where shallow water tables remain for a longer period of time (Voesenek et al. 2004). Only under such prolonged shallow flooding, shoot elongation feasibly leads to projecting leaves above the water table and appears to be an effective remedy to counteract the problems of oxygen deficiency.

Flooding is often too deep to restore air contact by shoot elongation, but plants have shown to be able to withstand the stress imposed by full submergence. Our study has elucidated the responses of SLA and aerenchyma content to be determinants of plant performance under such conditions. These traits have been identified as representatives of two independent trait clusters.

It is tempting to relate these two trait clusters to ecological conditions of partial or full submergence. Under conditions of shallow flooding, increased plant height and aerenchyma content might be most important, as they restore air contact and aerate the roots respectively. Plants growing in deeply flooded habitats, however, will benefit more from increased SLA and thus from underwater gas exchange capacity rather than increased plant height, as elongation growth would be only associated with costs but not with benefits in deep water (Setter and Laureles 1996). However, enhanced within-plant gas diffusion by aerenchymatous tissue will not only be of importance under shallow flooding, but also under complete submergence. Moreover, an increased SLA will already be beneficial in the time span preceding air contact restored by height growth, as well as under conditions in which the water column is too high for leaves to reach the surface. The differences between the ecological conditions of flooding thus do not seem to be so clear cut. Several species from wet habitats, such as *Rumex palustris* are well able to both, escape the water column through petiole elongation and increase gas exchange capacity of their leaves (Mommer et al. 2004), suggesting a bet-hedging strategy. The exact benefits of trait expression will depend on the frequency, depth and duration of the flood and this risk-spreading will, therefore, be advantageous in such dynamic and unpredictable environments as floodplains.

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Chapter 3

A functional comparison of acclimation to shade and submergence in two terrestrial plant species

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Abstract

Terrestrial plants experience multiple stresses when they are submerged, caused by both oxygen deficiency due to reduced gas diffusion in water, and by shade due to high turbidity of the floodwater. It has been suggested that responses to submergence are *de facto* responses to low light intensity. We investigated to what extent submergence and shade induce similar acclimation responses by comparing two terrestrial *Rumex* species that differ in their responses to flooding.

Our study confirms that there are strong similarities between acclimation responses to shade and submergence. Petiole length, specific leaf area (SLA), chlorophyll parameters and underwater light compensation points changed at least qualitatively in the same direction. Maximum underwater photosynthesis rate, however, did discriminate between the functionality of the responses, as the acclimation to submergence showed to be more effective than acclimation to shade at saturating light. We conclude that acclimation to submergence involves more than an increase in SLA to achieve the significant reduction of diffusion resistance for gas exchange between leaves and the water column, and probably also involves a decrease in cuticle resistance.

Introduction

Plant growth is affected by multiple environmental factors operating simultaneously (Chapin *et al.*, 1987). Submerged terrestrial plants growing in river floodplains experience at least two factors limiting plant performance, i.e., reduced gas exchange, resulting in oxygen deficiency within the plant (Armstrong *et al.*, 1994; Vartapetian & Jackson, 1997; Blom, 1999), and low light intensities since flood water is usually highly turbid (Vervuren *et al.*, 2003).

It has been suggested that responses of terrestrial plants to submergence are in fact shade responses because acclimation to shade and submergence results in similar morphologies, e.g., elongation of petioles (compare Voesenek *et al.* (2004) to Smith & Whitelam, (1997)) and vertical orientation of leaves (hyponasty; compare Cox *et al.* (2003) to Pierik *et al.*, (2003)). Also for submerged aquatic macrophytes, the morphological and physiological characteristics of submerged leaves are often interpreted as shade adaptations (Bowes & Salvucci, 1989; Frost-Christensen & Sand-Jensen, 1995; Wells & Pigliucci, 2000; Boeger & Poulson, 2003). However, others have suggested that this presumed shade morphology of aquatic plants is a secondary result of optimising gas exchange capacity (Nielsen & Sand-Jensen, 1989; Madsen & Maberly, 1991).

It should be realized that different environmental stimuli may induce similar morphological modifications of leaves (Arntz & Delph, 2001; Meinzer, 2003) and roots (Schmidt & Schikora, 2001). For example, the morphology of leaves that are shaded or submerged may appear the same. However, these leaves may have different functional characteristics in either situation. To distinguish if an apparent trait similarity is also functionally the same, comparative in-depth studies are needed to gain understanding of the complex relationship between environmental signals and the resulting phenotypes.

In this study we explicitly investigate to what extent the acclimation responses to shade and submergence are convergent in terrestrial floodplain species. Two terrestrial *Rumex* species that are different in their ability to respond to submergence (Nabben *et al.*, 1999) allowed us to investigate whether these differential responses to submergence are consistent with their responses to shade. First, we investigated responses to shade, including typical shade acclimation of photosynthesis (lower maximum photosynthesis rates, lower dark respiration and lower light compensation points (Sims & Pearcy, 1994; Terashima & Hikosaka, 1995; Givnish *et al.*, 2004) as well as well-known morphological (specific leaf area (SLA) and petiole length) and biochemical (chlorophyll content and a/b ratio) parameters (Björkman, 1981; Givnish, 1988; Terashima *et al.*, 2001). Next we compared these morphological and biochemical responses to shade with those under water. If these responses to submergence are in fact shade responses, we expect changes under water to mimic the changes in shaded drained conditions, irrespective of the light regime under water. Finally, we investigated underwater photosynthesis of plants grown under four different conditions, i.e., every combination of drained or submerged with high or low light. If responses to submergence are in fact shade responses, we expect drained shade acclimated plants to have similar underwater photosynthesis characteristics as submergence acclimated plants. If gas diffusion is more limiting for plant growth under water than light (Bowes, 1987; Maberly & Madsen, 2002), submergence acclimated plants will behave similarly under both light conditions under water, whereas drained plants -shade acclimated or not- will do worse.

Materials and methods

Plant material

Two floodplain species, *Rumex palustris* Sm. and *R. thyrsiflorus* Fingerh., were studied in this experiment. *R. palustris* is a characteristic species for the low elevated and thus frequently flooded sites of the floodplain, whereas *R. thyrsiflorus* occurs at drier and higher elevations (Nabben *et al.*, 1999). The species differ in resistance to submergence, as *R. palustris* has a much higher survival time under water than *R. thyrsiflorus* (Nabben *et al.*, 1999). Furthermore, when submerged, *R. palustris* is able to develop leaves with a more elongated morphology than the leaves that are formed in air, whereas the leaves of *R. thyrsiflorus* do much more resemble each other. Seeds of these species were collected from the floodplains near Nijmegen (the Netherlands) and germinated for 10 days on moistened filter paper in petri dishes at temperatures of 22°C during daytime (Photosynthetic Photon Fluence Rate (PPFD) 20 µmol m^{-2} s^{-1}) and 10°C at night. The seedlings were transplanted to pots of 6x6x8 cm, containing a mixture of one volume potting soil and one volume sand. Plants were further grown for 20 days (on average, see below) in a growth chamber (PPFD at leaf level approx. 220 µmol m^{-2} s^{-1} (sodium lamps SON-T plus 600 W and fluorescent light TLD Reflex 36W/840R, Philips, Eindhoven, the Netherlands); day/night cycle 16h light/8h dark; temperature 20±2°C). The positions of the pots were changed regularly to homogenize the growth conditions among replicate plants.

Experimental setup

In the first experiment, one group of plants was subjected to high or low light intensities (PPFD at leaf level 140 vs. 30 µmol m^{-2} s^{-1}; referred to as high light (HL) *versus* low light (LL)) under drained conditions. Aerial photosynthesis characteristics were determined on these plants.

A full factorial second experiment with the two factors, water level (drained vs. complete submergence) and light intensity (HL vs. LL) was performed with another group of plants. The plants treated under these conditions enabled morphological, biochemical and underwater photosynthesis comparisons of the acclimation to shade and submergence. Each treatment lasted 12 days, during which leaves present at the start of the experiment acclimated and 1-3 new leaves were formed in each treatment. Plants in submergence and drained treatments had different growth rates and, therefore, duration of the growth period previous to the treatment was chosen in such a way that plants reached a comparable developmental stage at the end of each treatment, based on the total number of leaves. The period from sowing until the start of drained and submerged treatments varied therefore from 26 to 34 days.

All treatments were carried out in opaque polyethylene basins (80x60x70 cm). The basins used the drained treatment were kept drained, whereas the basins used for submergence were filled with tap water. The water was circulated with a flow rate of 1.5 L min^{-1} and filtered to prevent algal growth. Temperature of the water was 20°C. Total dissolved inorganic carbon concentration, measured with an infra red gas analyser (Horiba PIR 2000; Northampton, UK), was 1.3 mM, which resulted at pH 8.4 in a free CO_2 concentration 15 µM. Neutral filters (type ULS 10, Ludvig Svensson, the Netherlands) were used to attain the two light intensities in the basins.

Aerial photosynthesis measurements (Experiment 1)

To verify if both species are able to photosynthetically acclimate to shade, we measured *aerial* photosynthesis characteristics on drained plants that had acclimated to high and low light intensity. Please note that these measurements were only performed on drained plants and not on submergence-acclimated plants. These measurements were performed on leaves (leaf number 7) of drained plants (n=4) by measuring CO_2 uptake using a portable infrared gas analyser (LI-COR 6400; Lincoln, Nebraska, USA) (370 µmol mol^{-1} CO_2, 80 % relative humidity, temperature 20 °C in leaf chamber).

Maximum photosynthesis rates were observed at a PPFD of 500 µmol m^{-2} s^{-1} for both treatments. Respiration rates were measured in dark. Apparent quantum, a parameter that expresses the initial slope of the photosynthesis-irradiance curve was calculated by linear regression of the net values obtained at PPFDs of 13, 27 and 40 µmol m^{-2} s^{-1}. Light compensation points were calculated from extrapolation of data from these same PPFDs to the light intensity where respiration equals assimilation and thus net photosynthesis is zero.

Morphological and biochemical parameters (Experiment 2)

Morphological responses to shade and submergence are reflected by petiole length and changes in SLA. The petiole length of the largest leaf was recorded (n=10), and the specific leaf area (SLA) was calculated from the total dry weight of three leaf punches (1.07 cm^2 each) per plant (n=6-8). This resulted in relatively high SLA values, as vein material was excluded from the samples.

Biochemical acclimation responses are reflected by changes in chlorophyll content and relative investment in chlorophyll a/b. Chlorophyll contents of the youngest full-grown leaves were determined spectrophotometrically (Shimadzu Benelux, Den Bosch, the Netherlands) after extraction with 96% ethanol (n=6-10). Equations of Wintermans & de Mots (1965) were used to calculate the chlorophyll a and b pigment concentrations.

Underwater photosynthesis measurements (Experiment 2)

Effects of photosynthetic acclimation to shade and submergence were investigated by measuring underwater photosynthetic characteristics, defined as oxygen production or consumption in water. Therefore, plants from all treatments were submerged in transparent Perspex cuvettes (820 or 1200 ml), depending on the size of the plants. The cuvettes were filled with 5.0 mM $NaHCO_3$, which was adjusted to pH 6.5 with 7.5% HCl, yielding a free CO_2 concentration of 2.2 mM. Before pH adjustment the O_2 concentration was lowered to 50% of air saturation by flushing with N_2 to ensure a steep diffusion gradient from the leaves to the surrounding water. Measurements were performed on whole shoots (n=6-8) of which the roots were excised. After one hour acclimation, oxygen release and uptake were recorded using an YSI oxygen probe electrode (type YSI 5331, Yellow Springs, Ohio, USA) connected to a Diamond Micro Sensor (Ann-Arbor, Michigan, USA). A rotary pump (Rena type C40, Annecy, France) provided a constant circulation of 10 L min^{-1} in order to minimize the development of boundary layers. Water temperature was kept constant at 20±1°C using a Haake DC 50 cooling system (Karlsruhe, Germany). Real-time logging of the oxygen concentration was performed with a Grant 1600 SQ data logger (Cambridge, UK). Average oxygen production was calculated from the logged data, after the system had stabilised for 15 minutes.

Underwater photosynthesis parameters were measured at the same light intensities as described above for the aerial photosynthesis measurements. Different light intensities in the underwater photosynthesis set-up (PPFD 500, 40, 27, 13 and 0 µmol m^{-2} s^{-1}) were achieved by attenuating the light (SON-T plus 600 W, Philips, Eindhoven, the Netherlands) with metal gauze screens. The cuvette with plant was placed horizontally and mirrors around the cuvette reflected the light coming from above so that self-shading was minimised. Dark respiration measurements were performed at 80% of air saturation, which is not limiting respiration in *Rumex* species (Laan *et al.*, 1990). After the underwater photosynthesis measurements, shoot dry weight and leaf area were determined. Shoot dry weight was determined after drying for 48 h at 80 °C. Leaf area was determined with a portable leaf area meter (LI-COR 3000, Lincoln, Nebraska, USA).

Table 1. Chlorophyll parameters of *R. thyrsiflorus* and *R. palustris* grown at high and low light intensities in drained and submerged conditions. Data are means ± SE, n= 6-10. Data were ln transformed prior to statistical analysis. Different letters in the columns within species indicate significant differences ($p<0.05$).

	Drained		**Submerged**	
	High light	Low light	High light	Low light
Rumex thyrsiflorus				
mg chlorophyll per m^{-2}	141 ± 7 ab	126 ± 6 a	158 ± 7 b	142 ± 4 ab
mg chlorophyll per g DW	7.2 ± 0.86 a	12.27 ± 0.47 b	10.37 ± 0.59 b	10.43 ± 0.47 b
chlorophyll a/b ratio	4.0 ± 0.28 a	2.69 ± 0.06 b	2.75 ± 0.10 b	2.85 ± 0.05 b
Rumex palustris				
mg chlorophyll per m^{-2}	127 ± 4 A	148 ± 3 A	144 ± 13 A	137 ± 4 A
mg chlorophyll per g DW	5.3 ± 0.40 A	18.6 ± 1.65 B	15.3 ± 1.49 B	14.07 ± 0.47 B
chlorophyll a/b ratio	3.4 ± 0.05 A	2.7 ± 0.03 B	3.13 ± 0.05 C	2.8 ± 0.05 B

Statistical analysis

Within a species, the data were analysed with two way ANOVA and Scheffé post hoc tests across different levels of light intensities and water levels with significance levels set to 0.05. Data were ln transformed in several cases in order to have equal variances. Differences between aerial photosynthesis parameters of drained HL and LL plants were analysed using Student's T-tests (p=0.05).

Results

Responses to shade (Exp. 1&2)

Both *Rumex palustris* and *R. thyrsiflorus* showed classic shade responses under drained conditions for all investigated parameters. The aerial photosynthesis measurements (Exp. 1) indicated that both species show entirely normal shade acclimation. As expected, maximum photosynthesis rates were reduced and accompanied by low dark respiration rates in response to low light (Table 1). Furthermore, light compensation points tended to decrease and apparent quantum yields were unaffected (Table 1).

Typically, petiole length (Fig. 1, Exp. 2) and the specific leaf area (SLA; Fig. 2; Exp. 2) increased significantly under shaded conditions compared to high light conditions. As expected, shade acclimation under drained conditions did not reveal significant differences in chlorophyll content per unit surface area, but expressed per unit dry weight shade acclimated plants contained significantly more chlorophyll than high light grown plants (Table 2, Exp. 2). Chlorophyll a/b ratios decreased significantly under shaded conditions (Table 2), indicating a relative higher investment in light harvesting capacity than in carbon-assimilating capacity.

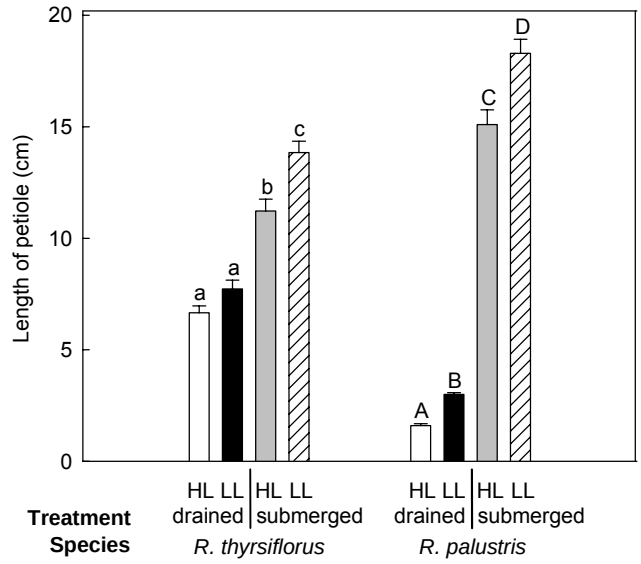

Figure 1. Petiole length of the largest leaf of *R. thyrsiflorus* and *R. palustris* plants grown at high (HL) or low (LL) light intensities in drained or submerged conditions. Data are means ± SE, n=10. Data were ln transformed prior to statistical analyses. Bars with different letters within each species are significantly different ($p<0.05$).

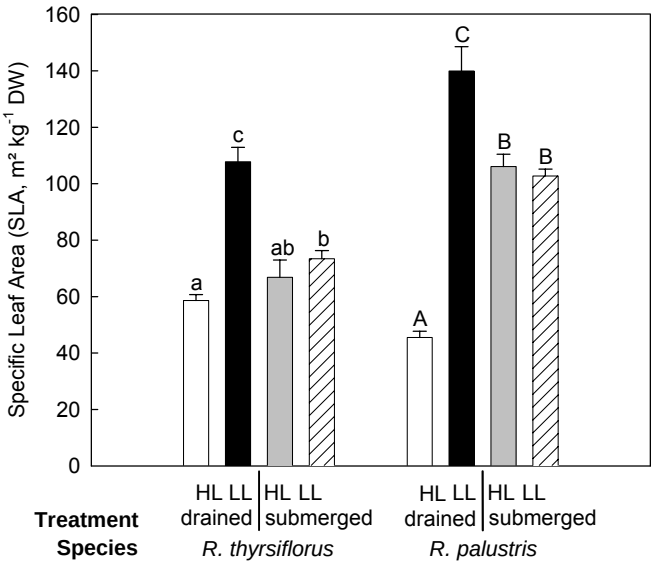

Figure 2. Specific leaf area of *R. thyrsiflorus* and *R. palustris* plants grown at high (HL) or low (LL) light intensities in drained or submerged conditions. Values were obtained from the weight and surface area of leaf punches. Data are means ± SE, n=6-8. Data were ln transformed prior to statistical analyses. Bars with different letters within each species are significantly different ($p<0.05$).

Responses to submergence (Exp. 2)

Both species showed significant petiole elongation upon submergence, when high light plants from the submerged treatment were compared with drained high light plants (Fig. 1,

Table 2. Aerial photosynthesis parameters of drained *R. thyrsiflorus* and *R. palustris*, grown at high and low light intensities. P_{max} is the maximum net photosynthesis rate at PPFD of 500 µmol m^{-2} s^{-1} (µmol CO_2 m^{-2} s^{-1}), R_d is dark respiration rate (µmol CO_2 m^{-2} s^{-1}), α is the apparent quantum yield calculated by linear regression of the net values obtained at PPFDs of 13, 27 and 40 µmol m^{-2} s^{-1} (µmol CO_2 $µmol^{-1}$ photons), light compensation points (LCP) were calculated from extrapolation of data from these same PPFDs (µmol photons m^{-2} s^{-1}). Data are means ± SE, n=4. Student's T tests were performed to distinguish shade effects. Significance levels: *** p<0.001, ** p<0.01, * p<0.05, n.s. not significant.

	High light	Low light	
Rumex thyrsiflorus			Sign.
P_{max}	8.41 ± 0.438	5.42 ± 0.385	**
R_{dark}	0.56 ± 0.051	0.39 ± 0.022	*
α	0.077 ± 0.014	0.077 ± 0.018	n.s.
LCP	5.8 ± 0.6	2.6 ± 1.5	n.s.
Rumex palustris			
P_{max}	8.03 ± 0.203	5.49 ± 0.194	***
R_{dark}	0.39 ± 0.037	0.20 ± 0.054	*
α	0.070 ± 0.004	0.063 ± 0.009	n.s.
LCP	7.0 ± 1.3	3.2 ± 1.5	n.s.

significant main water level effect, Table 3). Low light under water induced additional elongation (Fig. 1). However, the petioles of *R. palustris* elongated under water several fold more than those of R*. thyrsiflorus*.

SLA increased in response to submergence, although this increase was less pronounced than in shade (Fig. 2). Light conditions under water did not affect the change in SLA (Fig. 2). The chlorophyll parameters changed in a similar way in response to submergence as in response to low light (significant interaction LxW for chlorophyll per leaf area and chlorophyll a/b ratio Table 2,3).

Underwater photosynthesis measurements (Exp. 2)

The typical differences in aerial light response curves that were observed between high and low light grown drained plants, were not apparent in underwater photosynthesis measurements. Maximum net photosynthesis rates under water were similar for drained high and low light plants of both species (Fig. 3). Apparently, shade acclimation has no detectable effect on photosynthesis under water.

A strong change in underwater photosynthesis rate, however, was observed in the submergence acclimated plants: these plants showed significantly higher maximum

Table 3. Results of ANOVA analyses across levels of light intensity (L) and water level (W) and their interaction for both *Rumex* species. SLA is specific leaf area (m^2 g^{-1} DW), P_{max} is the maximum net photosynthesis rate under water (µmol m^{-2} s^{-1}), R_d is dark respiration rate under water (µmol m^{-2} s^{-1}), LCP is light compensation point under water (µmol m^{-2} s^{-1}), α is the apparent quantum yield under water (µmol $µmol^{-1}$). Significance levels: *** p<0.001, ** p<0.01, * p<0.05, n.s. not significant.

Parameter	**Effect**	*R. thyrsiflorus*	*R.palustris*
Petiole length	L	***	***
	W	***	***
	LxW	n.s.	***
SLA	L	***	***
	W	*	***
	LxW	***	***
mg chlorophyll per m^{-2}	L	*	n.s.
	W	*	n.s.
	LxW	n.s.	n.s.
mg chlorophyll per g DW	L	***	***
	W	n.s.	***
	LxW	**	***
chlorophyll a/b ratio	L	***	***
	W	**	n.s.
	LxW	***	***
Underwater P_{max}	L	n.s.	***
	W	***	n.s.
	LxW	n.s.	***
Underwater R_{dark}	L	n.s.	n.s.
	W	n.s.	n.s.
	LxW	n.s.	***
Underwater LCP	L	n.s.	***
	W	**	*
	LxW	n.s.	*
Underwater α	L	*	n.s.
	W	n.s.	n.s.
	LxW	n.s.	***

photosynthesis rates than drained ones, except for submerged low-light *R. palustris*, in which the maximum photosynthesis rate was lower (Fig. 3).

Light compensation points (Fig. 4) were highest in the high light drained plants in both species and were significantly lower in low light drained and submerged plants. Dark respiration (Fig. 3) and apparent quantum yield (Fig. 5) were not affected by the treatments in *R. thyrsiflorus*. In contrast with *R. thyrsiflorus*, these parameters changed significantly in *R. palustris* (Fig. 3, 5): submerged high light plants had lower respiration rates and higher apparent quantum yields than the drained plants, which may contribute to survival under water. Surprisingly, submerged low light *R. palustris* plants showed opposite responses.

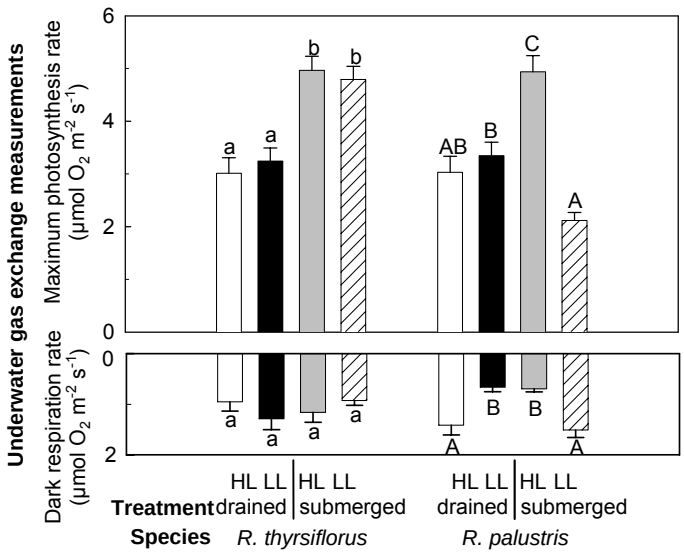

Figure 3. Underwater maximum photosynthesis rate and dark respiration of *R. thyrsiflorus* and *R. palustris* plants grown at high (HL) or low (LL) light intensities in drained or submerged conditions. Data are means ± SE, n=6-8. Data were ln transformed prior to statistical analyses. Bars with different letters within each species are significantly different ($p<0.05$).

Discussion

In the literature, acclimation to submergence has been hypothesized to be in fact acclimation to shade (Bowes & Salvucci, 1989; Frost-Christensen & Sand-Jensen, 1995; Wells & Pigliucci, 2000; Boeger & Poulson, 2003). Our results underlined this contention since the morphological and biochemical responses to shade and submergence were in the same direction, although they could differ by an order of magnitude. Moreover, underwater light compensation points were lower in both shade acclimated and submergence acclimated plants as compared to drained high light grown plants. The difference between the acclimation responses, however, became clear when measuring underwater photosynthesis at saturating light intensities. Maximum underwater photosynthesis rates were much higher in submergence acclimated than in shade acclimated plants suggesting that acclimation to submergence is directed towards optimisation of gas exchange, rather than to optimisation of light capture.

Morphological and biochemical responses to shade and submergence are similar

Many of the morphological responses to shade and submergence were at least qualitatively the same. Still, significant interactions between light and water level were observed for petiole length and specific leaf area (SLA) in both *Rumex* species (Fig. 1,2, Table 3). Submergence affected petiole length significantly more than shade, which may be explained by mechanical support from the water column, allowing longer petioles to emerge under

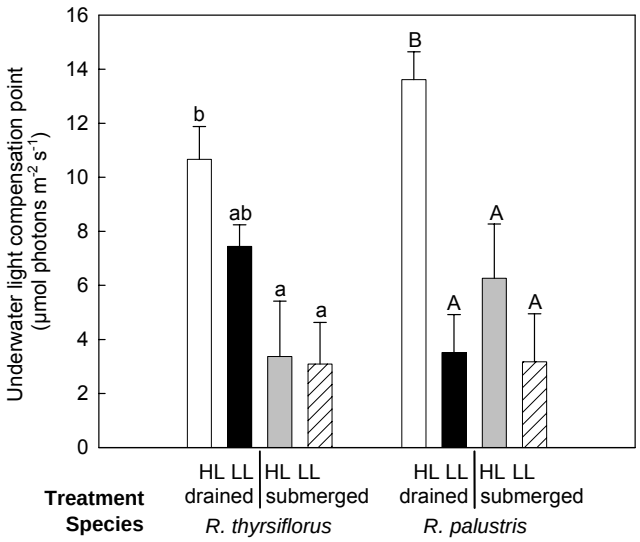

Figure 4. Light compensation points under water of *R. thyrsiflorus* and *R. palustris* plants grown at high (HL) or low (LL) light intensities in drained or submerged conditions. Data are means ± SE, n=6-8. Bars with different letters within each species are significantly different ($p<0.05$).

water. In contrast to petiole elongation, SLA was affected more strongly by the shade treatment.

Chlorophyll content and a/b ratio responded to submergence like under low light, again underlining the presumed similarity between the responses. Chlorophyll contents on a leaf area basis was similar for drained and submerged plants and contrast with the aquatic literature, where chlorophyll content is generally lower in aquatic leaves of amphibious plants

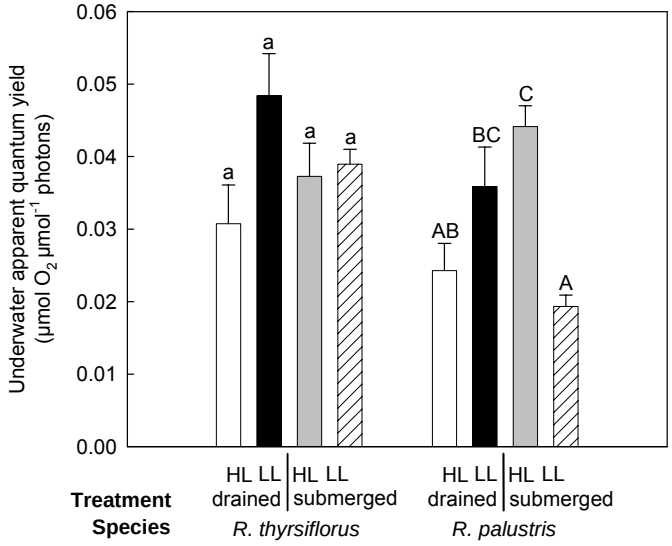

Figure 5. Under water photosynthetic efficiency of *R. thyrsiflorus* and *R. palustris* plants grown at high (HL) or low (LL) light intensities in drained or submerged conditions, expressed as apparent quantum yield. Data are means ± SE, n=6-8. Bars with different letters within each species are significantly different ($p<0.05$).

than in their aerial counterparts (Sand-Jensen *et al.*, 1992; Nielsen, 1993; Frost-Christensen & Sand-Jensen, 1995; Nielsen & Sand-Jensen, 1997).

Acclimation to shade and submergence results in similar underwater light compensation points

Acclimation to shade and submergence resulted in the same efficient use of low light intensities, since low underwater light compensation points were lower in shade and submergence acclimated plants compared to high light drained plants (Fig. 4). The light compensation point is determined by the amount of respiratory CO_2 release in photorespiration and dark respiration as compared to CO_2 fixation. For lower respiratory carbon losses, less CO_2 fixation and thus less light absorption is required to reach net positive photosynthesis. Plants with lower CO_2 compensation points are thus expected to show lower respiration rates, and that is what we observed for submergence acclimated *R. palustris* plants grown in high light. Respiratory costs were also lower in shade acclimated plants of both species when measured in air (Table 1), but under water this could only be detected in shade acclimated *R. palustris* plants. In addition, the decreased light compensation points will also have resulted from increased light harvesting capacity in shade acclimated and submergence acclimated plants compared to drained high light plants since acclimation reduced the chlorophyll a/b ratio.

Maximum underwater photosynthesis distinguishes shade and submergence

Underwater photosynthesis measurements at saturating light discriminated between acclimation responses to shade and submergence. Acclimation to submergence was far more beneficial for underwater photosynthesis than acclimation to shade, as submergence-acclimated plants showed higher maximum rates of under water photosynthesis compared to the drained plants of either light intensity (Fig. 3). Surprisingly, the maximum underwater photosynthesis rate of both high and low light acclimated drained plants was similar (Fig. 3), whereas the aerial photosynthesis rates were much lower in the shade-treated plants (Table 1). Typically, shade acclimated plants have reduced maximum photosynthesis rates in air (Table 1; (Björkman, 1981; Givnish, 1988) and the fact that this was not apparent when photosynthesis was measured under water suggests that other components of shade acclimation can be beneficial for maximum photosynthesis rate under water (Fig. 3).

The beneficial effect of shoot acclimation to submergence – and to a lesser extent acclimation to low light intensity – is likely to originate from a reduction in diffusion resistance to gas exchange between leaves of plants and their aquatic environment, analogous to aquatic plants (Nielsen, 1993; Frost-Christensen & Sand-Jensen, 1995). An increased SLA indicates that the relative surface area that is in contact with the water layer is larger. Under

water, gases enter the plant via cuticle and epidermis cells and thus increased gas exchange area will increase gas exchange capacity. The drained shaded plants showed the highest SLA values, but this resulted in lower rather than higher maximum photosynthesis rates under water compared to submergence-acclimated plants. This suggests that acclimation to submergence involves more than simply the increased SLA observed in low light treated drained plants. Since the major limitation for gas diffusion between the plant and the water layer is likely to be the cuticle (Frost-Christensen *et al.*, 2003), effective acclimation to submergence is most probably due to a reduced cuticle resistance (Mommer *et al.*, 2004).

Underwater photosynthesis at saturated CO_2 does not explain flooding tolerance

Of the two terrestrial *Rumex* species that we investigated, the flood tolerant *R. palustris* showed the highest phenotypic plasticity of the two species to both submergence and shade with respect to shoot morphological parameters (Fig. 1, 2) and chlorophyll content (Table 2). It remains to be elucidated whether this higher plasticity of *R. palustris* leads to higher fitness under submerged conditions, because the difference in flooding tolerance with the less tolerant *R. thyrsiflorus* was neither associated with higher maximum underwater photosynthesis rates nor by lower light compensation points or increased apparent quantum yields. Clearly both species photosynthesised equally well in this experiment with unlimited supply of CO_2. Earlier attempts to relate underwater photosynthesis to flooding tolerance failed as well, as non-acclimated plants were investigated at high CO_2 concentrations (Voesenek *et al.*, 1993; He *et al.*, 1999; Vervuren *et al.*, 1999). We, therefore, suggest that it is not the photosynthesis capacity under water *per se* that determines survival under water, but photosynthetic performance under more natural conditions, where CO_2 availability is limited and thus low gas diffusion resistance becomes more important.

No additional shade acclimation under water

Low light conditions did not induce additional responses under water with the notable exception of petiole elongation as for this parameter significant differences were observed between the two light treatments under water. Most effects of submergence overruled the effect of light intensity (*cf.* Dale & Causton (1993) and Lenssen *et al.* (2003)), suggesting a hierarchy in effects. However, light acclimation can take place underwater in real aquatic plants species with respect to SLA (Spence *et al.*, 1973; Goldsborough & Kemp, 1988) and plant height or internode length (Spence & Chrystal, 1970; Chambers & Kalff, 1985). This implies that the hierarchical effect of submergence over shade may depend on the tolerance of a species to submergence, as aquatic plant species have adapted to an aquatic life in such a way that gas exchange is not a stress factor (Lenssen *et al.*, 2003).

Unexpectedly, the shade acclimated submerged *R. palustris* plants showed the lowest maximum photosynthesis rate under water and relatively high dark respiration rates (Fig. 3). A possible explanation, frequently proposed in aquatic photosynthesis studies, is that the rather high CO_2 concentration in the underwater measurements may have had a depressing effect on the maximum photosynthesis rate (Weber *et al.* 1979, Allen & Spence, 1981, Denny *et al.*, 1983; Pokorný *et al.*, 1985) and even may have damaged cell components. Since *R. palustris* was likely to attain the highest rate of CO_2 influx of the two species, because it showed the strongest morphological responses (Fig. 1,2), high CO_2 concentrations may specifically have affected maximum photosynthesis in this species. This may have been particularly relevant for the low-light submerged plants, because these shaded plants will have experienced a relatively low energy status. The relatively high dark respiration rates may indicate higher costs allocated to repair mechanisms in these plants.

Concluding remarks

Our study shows that there is indeed strong similarity between acclimation responses to shade and submergence. Maximum underwater photosynthesis rate, however, did clearly discriminate between the functionality of the responses. This suggests that acclimation to submergence involves at least partly different processes than acclimation to shade, the first being predominantly directed to increased gas exchange capacity rather than to light capture. This study illustrates that morphological and biochemical responses to environmental cues need to be studied in depth before their physiological functionality can be understood.

It remains intriguing, however, that morphological responses are so convergent whereas the functional consequences are clearly different. This indicates that initially different signals (shade vs. submergence) may converge later in the signal transduction cascade to similar responses. At the same time, other responses are induced that are unique for shade or submergence, as reflected in the underwater maximum photosynthesis rates. It will be a long way to go before we understand how low light and low CO_2 availability induce at least partly the same morphology of a plant, but the expected low assimilate levels may sure be a link between both acclimation responses.

Acknowledgements

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Chapter 4

Acclimation of a terrestrial plant to submergence facilitates gas exchange under water

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Abstract

Flooding imposes stress upon terrestrial plants since it severely hampers gas exchange rates between the shoot and the environment. Resulting oxygen deficiency is considered to be the major problem for submerged plants.

Oxygen microelectrode studies have, however, shown that aquatic plants maintain relatively high internal oxygen pressures under water, and even may release oxygen via the roots into the sediment, also in dark. Based on these results, we challenge the dogma that oxygen pressures in submerged terrestrial plants immediately drop to levels at which aerobic respiration is impaired.

Our study demonstrates that the internal oxygen pressure in the petioles of *Rumex palustris* plants under water is indeed well above the critical oxygen pressure for aerobic respiration, provided that the air-saturated water is not completely stagnant.

We also show the beneficial effect of shoot acclimation of this terrestrial plant species to submergence for gas exchange capacity. Shoot acclimation to submergence involved a reduction of the diffusion resistance to gases, which was not only functional by increasing diffusion of oxygen into the plant, but also by increasing influx of CO_2, which enhances under water photosynthesis.

Introduction

Plants growing in flood prone environments experience submerged conditions during parts of their life cycle. For terrestrial species, such complete submergence imposes stress because gas exchange rates between the shoot and the environment are severely reduced, since the

diffusion rates of gases are 10^4 times lower in water than in air (Armstrong 1979). As a result, oxygen deficiency is considered to be the major factor negatively affecting survival and growth of submerged plants (Vartapetian and Jackson 1997; Voesenek et al. 2004), because it leads to energy deficits due to hampered aerobic metabolism (Crawford and Brändle 1996).

Many studies have investigated the responses of floodplain species that reduce the negative effects of oxygen deficiency (Armstrong et al. 1994a; Blom 1999). Several species are able to change their shoot morphology in such a way that contact with the air can be restored, for example by enhanced elongation and reorientation of the leaves (Voesenek et al. 2003a). Another common response of floodplain species is the increase of the amount of air spaces (aerenchyma) within the shoot and the roots to optimise within-plant gas transport (Colmer 2003). Furthermore, plants are able to switch to anaerobic metabolic pathways to prevent energy deficits and thus cell death (Perata and Alpi 1993).

Despite this broad palette of responses plants may employ to overcome the negative effects of submergence, oxygen pressures within the plant are considered to drop rapidly to very low levels when plants are submerged (Raskin and Kende 1983; Stünzi and Kende 1989; Rijnders et al. 2000). We have, however, reasons to challenge this dogma. This is inspired by recent gas exchange studies on aquatic plants showing that internal oxygen pressures in the shoots of these plants may in fact be sufficiently high to maintain aerobic respiration under natural conditions, even in the absence of photosynthesis (Greve et al. 2003). Pedersen et al. (1998) and Revsbech et al. (1999) were the first to show that submerged seagrasses and rice plants release oxygen into the sediment, during daytime conditions and, unexpectedly, also at night. The oxygen release during daytime was fed by leaf photosynthesis (Smith et al. 1984), and in dark by passive diffusion of oxygen from the water column via the aerenchymatous shoot into the roots (Pedersen et al. 1998; Pedersen et al. 2004).

Building on these results, we studied gas exchange rates between the shoot of a terrestrial plant and the surrounding water. We used *Rumex palustris*, a submergence resistant species (Nabben et al. 1999) as a model. This species allows us to investigate not only the default underwater gas exchange capacity of a non-acclimated terrestrial plant, but also whether shoot acclimation to submergence affects the underwater gas exchange capacity of the plant. *Rumex palustris* is a temperate floodplain species (Blom et al. 1994), which upon submergence shows typical wetland species characteristics, such as enhanced petiole elongation (Voesenek et al. 2004) and adventitious root and aerenchyma formation (Visser et al. 1996a). More importantly for the current study, this species also shows a high plasticity in leaf morphology upon submergence (Chapter 3). We expected that the submerged leaf morphology facilitates gas exchange under water. We therefore measured

not only the effect of the oxygen pressure of the water column on the internal oxygen pressure of the shoot, but also the contribution of under water photosynthesis to the internal oxygen pressure with varying CO_2 concentrations in the surrounding water.

Materials and methods

Plant material and treatments

Rumex palustris Sm. seeds were collected from the floodplains near Nijmegen (the Netherlands) and germinated for 10 days in a petri dish on moistened filter paper at temperatures of 22°C during daytime (Photosynthetic Photon Flux Density (PPFD) 20 µmol m^{-2} s^{-1}) and 10°C at night. Then the seedlings were transplanted to pots of 6x6x8 cm, containing a mixture of one volume potting soil and one volume sand and placed in a climate room (PPFD approx. 250 µmol m^{-2} s^{-1} (sodium lamps Philips SON-T plus 600W and fluorescent light TDL Reflex 36W/840R, Eindhoven, the Netherlands); the day/night cycle: 16h/8h; temperature 20°C) for 28 days.

To test the effect of shoot acclimation to submergence on internal oxygen pressure, one group of plants was then fully submerged, whereas the other group was kept drained. The submerged plants were flooded in a glass aquarium of 30x60x40 cm which was filled with tap water. Atmospheric air was bubbled through the water to maintain air-equilibrium concentrations of O_2 (21 kPa) and CO_2 (15 µM or 0.04 kPa). A pump (Rena, type 40C, Annecy, France) provided circulation of the water. In both treatments, PPFD was 140 µmol m^{-2} s^{-1}; the day/night cycle was 16/8 h, and the temperature 20°C. The treatments lasted 12-18 days, and the plants had developed at least two new leaves during the treatments.

Oxygen microelectrode measurements

Three aquaria containing 4.5 litre of water were placed in a thermostat-controlled bath to maintain the temperature at 20°C. The water in the aquaria consisted of partly de-ionised water with an alkalinity of 1.9 meq $litre^{-1}$ and a conductivity of 220 µS cm^{-1}. These two parameters together with pH and temperature, allowed the free CO_2 concentration in the water to be calculated according to Mackereth et al. (1978). A Clark-type oxygen microelectrode (OX10, Unisense, Århus, Denmark) placed in one of the aquaria monitored the oxygen pressure of the water surrounding the shoots. Desired O_2 and CO_2 concentrations of the water were achieved by bubbling the water with mixtures of O_2, CO_2 and N_2 obtained by mixing pure O_2, CO_2 and N_2 (Air Liquide, Ballerup, Denmark) using mass flow controllers (Brooks Instruments, Veenendaal, the Netherlands). Increasing CO_2 concentrations of the water concomitantly lowered the pH of the water from 8.5 at very low

CO_2 concentrations to 6.5 at the highest concentrations, but this pH range has been shown not to affect underwater photosynthesis in other amphibious plants (Andersen and Pedersen 2002; Sand-Jensen and Frost-Christensen 1998). Because of the bubbling, water was circulated in the aquaria with a flow velocity of 2-5 cm s^{-1}, which minimised the formation of boundary layers. Light was provided with glass fibre tubes attached to lamps (Osram Halogen Xenophot, Munich, Germany) with a PPFD of 450 µmol m^{-2} s^{-1}, which was sufficient to light-saturate underwater photosynthesis for this species (Mommer, unpublished)

Oxygen microelectrode measurements were performed in the petiole of the 5th developing leaf, which in all cases was formed during the treatment. Whole plants were mounted horizontally in the aquaria in such a way that the petiole was fixed 3 mm below the lamina and water moved freely around all lamina. Before transfer, roots with soil were gently transferred from the pot into a plastic bag (10x12 cm) with a piece of raw cotton around the root-shoot junction preventing the soil from being flushed out during the measurements. The setup was chosen in such a way that the lamina could be considered as the oxygen source in light because of photosynthesis, but also in dark, because of passive oxygen diffusion from the water layer into the leaf blades. This passive diffusion from the water into the plant mainly occurred through the lamina rather than through the petiole surface, since the lamina represents a much larger gas exchange area with a considerably higher surface area:volume ratio. The roots acted as a sink for internal oxygen, because they were placed in oxygen-deficient soil and were not in direct contact with the surrounding water.

Oxygen microelectrodes are nowadays widely used in plant sciences to measure oxygen pressures in tissues (Revsbech 1989, Armstrong 1994, Pedersen et al. 2004). The conical tip of the microelectrodes used in this study (OX10, Unisense, Denmark) was so small (ø=10 µm) that it could easily be inserted into the petiole without damaging the plant tissue. *In situ* partial oxygen pressures within the plant's petiole can thus be monitored very accurately, since the response time of these electrodes is fast (90% stability within <3 s) and their sensitivity to stirring so small (<2%) that their oxygen consumption is negligible. Data were logged from a picoamp meter (PA8000, Unisense) every 15 sec with an a/d converter (AD16, PicoTech, St. Neots, UK). Electrode signals were converted to oxygen concentrations using individual calibrations between 0 and 100% of air saturation before and after each series of measurements. Measurements were replicated three times.

Plant parameters

Plant parameters measured included the area (LI-COR 3000, Li-Cor Inc., Lincoln, NE, USA) of the leaf at which oxygen measurements were conducted, total leaf area of the plant, and dry weights of the lamina, petioles and roots, which were determined after drying for 48 h at 80 °C (n=6). Aerenchyma content of the petiole, a measure for within-plant gas transport

Table1. Plant parameters of non-acclimated and submergence-acclimated plants (means ± SE), statistically tested with Student's T-tests (*** p<0.001, n.s. not significantly different). Number of replicates is 6, except for aerenchyma content (n=10).

Parameter	Non-accl. plant	Accl. plant	Sign.
Petiole length (mm)	13 ± 1.0	47±2.0	***
Aerenchyma content of petiole (%)	20.3±0.4	29.5±0.9	***
Number of leaves	5.2±0.3	4.7±0.2	n.s.
Total dry weight (g)	0.22±0.01	0.22±0.02	n.s.
Area of leaf 5 (cm^2)	3.9±0.2	4.4±0.2	n.s.

capacity, was measured using the microbalance method as described in Visser and Bögemann (2003) (n=10). Differences between submergence-acclimated and non-acclimated plants were statistically analysed with Student's T-tests ($p<0.05$). If needed, data were ln-transformed prior to analysis in order to have equal variances.

Results

Plant parameters

The effect of submergence on plant morphology was clearly visible (Table 1). As expected, petioles of the submergence-acclimated plants were significantly larger compared to the non-acclimated controls. The aerenchyma content of the petiole tissue was also significantly higher in the submergence-acclimated plants (Table 1), indicating an increased within-plant transport capacity. The differences in porosity and length of diffusion pathway between the leaf types did not influence our microelectrode data, because the microelectrodes were inserted very close to the lamina, and thereby as close to the oxygen source as possible. Total number of leaves and total biomass remained unaffected upon submergence and the area of leaf 5 was not significantly different between the plant types either.

Diffusion of O_2 into the plant in the dark

We examined the relationship between the oxygen pressure of the water and the oxygen pressure within the plant when submerged (Fig. 1). The plants were submerged in the dark in this experiment, to be certain that only passive diffusion of oxygen from the water column into the plant occurred. Internal oxygen pressures reached a steady state within half an hour after manipulation of the oxygen pressure of the water in which the plants were measured, which was mainly due to the time needed to reach the desired gas pressure in the water. Internal oxygen pressures were proportional to the oxygen pressure in the water in both submergence-acclimated and non-acclimated plants, but still lower than the oxygen pressure

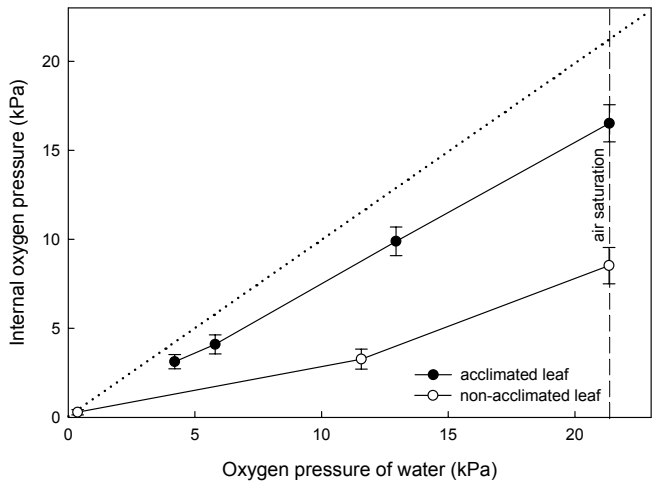

Figure 1. Relationship between oxygen pressure within petioles of submergence-acclimated (closed symbols) and non-acclimated (open symbols) *R. palustris* plants and the oxygen pressure of the surrounding water column in dark (PPFD = 0 μmol m^{-2} s^{-1}). Dotted line indicates the oxygen pressure of the water. Dashed line indicates the oxygen pressure of water column when air saturated. Data are means ± SE, n=3.

of the water column (Fig. 1). In air-saturated water (oxygen pressure 21 kPa) submergence-acclimated plants reached stable internal oxygen pressures of 17 kPa, whereas the non-acclimated plants only reached 9 kPa. The effect of shoot acclimation to submergence on gas exchange between the water column and the lamina was thus substantial: the internal oxygen pressure was almost a factor 2 higher in the ubmergence-acclimated plants compared to the non-acclimated plants.

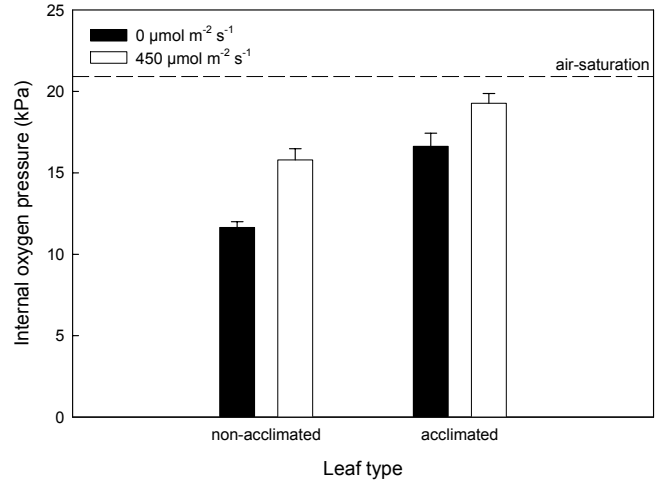

Figure 2. Internal oxygen pressures of petioles of non-acclimated and submergence-acclimated *R. palustris* plants in dark (PPFD = 0 μmol m^{-2} s^{-1}) and light (PPFD = 450 μmol m^{-2} s^{-1}). Free CO_2 concentration of the water is low (< 8 μM or 0.02kPa), the oxygen pressure of the water is air-saturated (21 kPa; dashed line). Data are means ± SE, n=3.

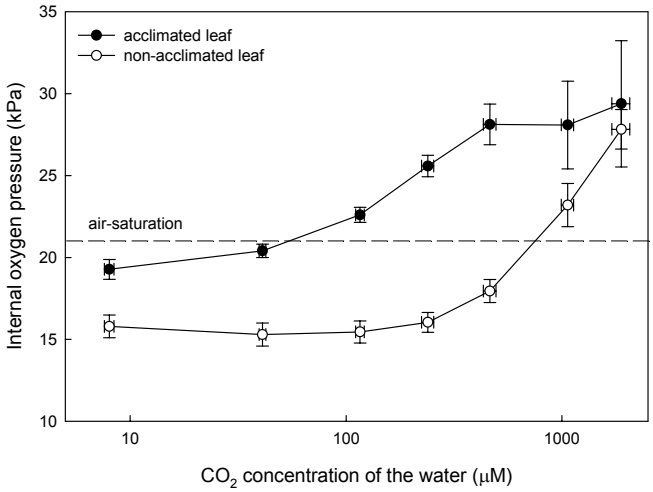

Figure 3. Internal oxygen pressures within petioles of submergence-acclimated (closed symbols) and non-acclimated (open symbols) *R. palustris* plants under conditions with increasing free CO_2 concentrations in the water column. The oxygen pressure of the water is air-saturated (21 kPa) (dashed line) and the PPFD = 450 μmol m^{-2} s^{-1}, which is saturating for underwater photosynthesis. Data are means ± SE, n=3.

Diffusion of CO_2 into the plant in the light

A significant increase of the internal oxygen pressure was observed in both non-acclimated and acclimated plants when the light was switched on after a dark period (Fig. 2), even though during this experiment the availability of free CO_2 in the water was rather low (free CO_2 concentration <8μM (<0.02 kPa)). The absolute increase in oxygen pressure in light compared to dark was 3.5 kPa and 5 kPa for the acclimated and non-acclimated plants respectively or, expressed as relative increase proportional to the dark oxygen concentration, 22 and 43 %.

When the free CO_2 concentration of the water increased (Fig. 3), internal oxygen pressures rose, indicating that photosynthetic rates increased. Submergence-acclimated plants responded already at 40 μM (0.1 kPa) free CO_2 in the water column, whereas non-acclimated plants were irresponsive till between 250 and 460 μM (0.65 -1.2 kPa) free CO_2 in the water column. At the latter concentration, internal oxygen pressures of submergence-acclimated plants were already saturated.

Discussion

Source-sink relation

Micro-electrode measurements across the submerged petiole showed that the exact depth of insertion of the microelectrode did not affect the readings of oxygen pressures (Fig. 4). Apparently, all cells within the petiole are located close to the intercellular air spaces, and

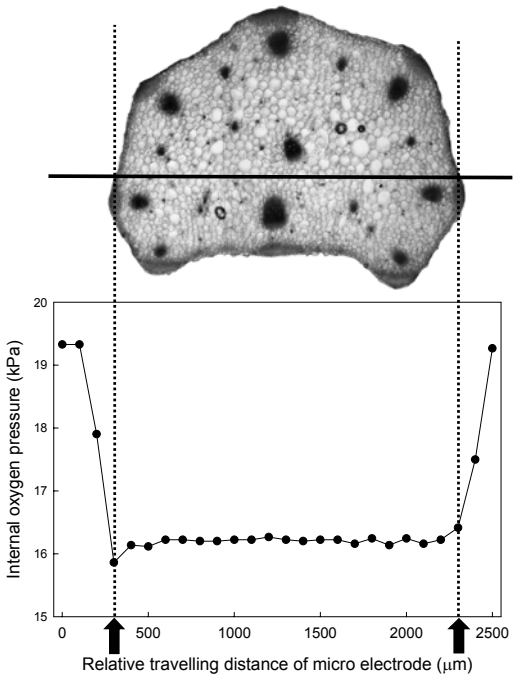

Figure 4. Representative profile of the internal oxygen pressures in a petiole of a submerged *Rumex palustris* plant, recorded with an oxygen microelectrode. Arrows indicate the point where the microelectrode entered or left the petiole.

these air spaces are sufficiently large (aerenchyma content of >20%) to provide a homogeneous oxygen concentration in radial direction and to prevent the occurrence of anaerobic cores in the petiole. Conversely, the internal oxygen pressure did decrease along the petiole with increasing distance from the lamina (Fig. 5), indicating that the lamina was the major source and the roots the major sink for oxygen within the plant. Unexpectedly, the relationship between internal oxygen pressure and distance from lamina was a linearly decreasing function (Fig. 5), which is in contrast with exponential predictions of gas diffusion models, deduced from experimental work with roots (Armstrong 1979; Armstrong 1994; Christensen et al. 1994). This may be explained by is the fact that the green petioles are also photosynthetically active and thus partly compensate for the decrease with distance.

Diffusion of oxygen into the plant in the dark

The internal oxygen pressure of the petioles of *R. palustris* plants correlated strongly with the oxygen pressure of the water column in dark (Fig. 1), which indicates that the water column functioned as an important source of oxygen for the plant. This passive diffusion of oxygen from the water column into the plant has been observed for aquatic macrophytes (Sand-Jensen et al. 1982; Pedersen et al. 1998; Greve et al. 2003; Pedersen et al. 2004), but to our knowledge, this is the first time to show this process in submerged terrestrial species. At

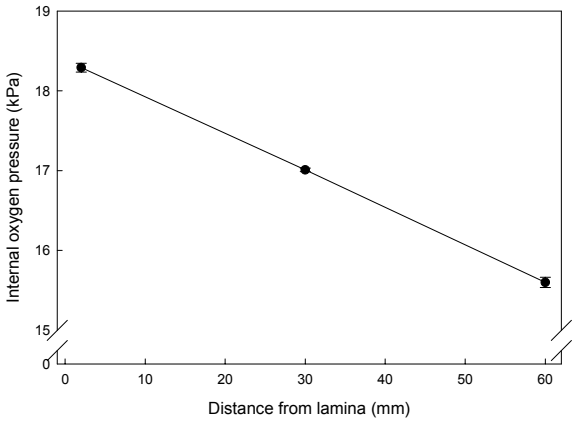

Figure 5. Internal oxygen pressures in the petiole of a submergence-acclimated *Rumex palustris* at different distances from the lamina in air-saturated water and PPFD of 450 μmol m^{-2} s^{-1}. Error bars originate from cross sectional profiles through the petiole (as in Fig. 4) as at each distance from the lamina the microelectrode traveled with steps of 100 μm through the petiole.

ambient oxygen pressure, the internal oxygen pressures of acclimated, but surprisingly also of non-acclimated *R. palustris* petioles were well above the critical internal oxygen pressure (0.8 kPa; Armstrong and Gaynard 1976; Armstrong and Webb 1985; Fig. 1) that is considered to be sufficient for maximum aerobic respiration.

Strikingly, the internal oxygen pressures of submergence-acclimated plants were considerably higher than those of non-acclimated plants (Fig. 1). Moreover, the internal oxygen pressures of petioles of submergence-acclimated plants were almost similar to the oxygen pressures of the water column. This shows clearly that shoot acclimation to submergence is particularly functional with respect to gas exchange capacity between the water column and the plant. The effect cannot be caused by a difference in gas exchange surface, as the leaves at which the measurements were conducted were not significantly different in leaf area between acclimated and non-acclimated plants (Table 1).

Our results contrast at first sight to the data presented by Raskin and Kende (1984), Stünzi and Kende (1989) and Rijnders et al. (2000), where internal oxygen pressures dropped to levels where aerobic respiration is impaired. The experiments of Rijnders et al. (2000) on the same species showed that the internal oxygen pressure of a non-acclimated plant dropped within 30 minutes from 21 to 4 kPa, and after 20 hours it was even less than 0.5 kPa. In the present experiment, within half an hour a stable oxygen pressure of 17 and 9 kPa was observed in the petioles of acclimated and non-acclimated plants respectively, when the oxygen concentration of the water was air-saturated. The difference between the experiments can be explained by a difference in the thickness of boundary layers around the leaves. In the present study we explicitly aimed at minimising boundary layer effects,

whereas in the other studies the solution surrounding the plant was not stirred, thereby leading to significant boundary layers around the leaves. True stagnant conditions are rare in the natural aquatic habitats occupied by amphibious plant species. Not only river systems, but even littorals of lakes are characterised by flow velocities that are sufficiently high to minimise boundary layer effects (Westlake 1967; Sand-Jensen and Pedersen 1999). This indicates that dark conditions are not necessarily as detrimental for the shoot under water as proposed in the former studies (Raskin and Kende 1984; Stünzi and Kende 1989; Rijnders et al. 2000).

Enhanced oxygen concentrations (Fig. 1, 2, 3) are not only of great importance for the energy status of the shoot, but also for the roots. The oxygen concentration at the shoot base determines the aeration status of the roots (Connell et al. 1999, Sorrell et al. 2000) if these organs are interconnected by aerenchymatous tissue (Armstrong et al. 1994b), which is the case in *R. palustris* (Laan et al. 1990). *R. palustris* plants increased the amount of aerenchymatous tissue in the petioles by 50% upon submergence (Table 1), indicating a high gas transport capacity within the acclimated plant (Colmer 2003). It is, therefore, likely that also the roots of the tolerant *R. palustris* will benefit from the acclimation of the shoot to the water environment.

Fixation of respired CO_2

Light appeared to be beneficial for *R. palustris* plants even at free CO_2 concentrations in the water that are below concentrations that can increase the internal oxygen pressure, as switching on the light rapidly raised the internal oxygen concentration with 22-43 % (Fig. 2). Because *R. palustris* cannot use HCO_3^- as carbon source for photosynthesis under water (data not shown, *cf. R. maritimus* and *R. crispus*; Laan and Blom (1990)), we suggest CO_2 produced during respiration in the light to be the carbon source that caused the increase of internal oxygen pressure in this experiment, although CO_2 uptake from the soil cannot be excluded. Respiration provides a constant delivery of CO_2 and fixation of this internally produced CO_2 may well contribute to photosynthesis leading to increased internal oxygen pressure in the light. That the putative fixation of respired CO_2 might reduce the loss of carbon in the submerged plant considerably is nicely illustrated by experiments of Nabben et al. (1999) and Vervuren et al. (2003). They showed that the availability of light under water is beneficial for survival and biomass maintenance of flooded terrestrial plants, including *R. palustris*, when free CO_2 in de water column was almost as low as in our experiment.

Diffusion resistance to CO_2

Increased external free CO_2 led to elevated internal oxygen pressures through enhanced photosynthesis in both acclimated and non-acclimated plants (Fig. 3). The internal oxygen

pressure was already higher in submergence-acclimated plants compared to the non-acclimated plants at the lowest CO_2 concentration, because of the earlier mentioned difference in diffusive oxygen flux from the water column into the plant (Fig. 1). Interestingly, internal oxygen pressures in submergence-acclimated leaves already increased at lower external CO_2 concentrations than in non-acclimated leaves. This provides additional proof that the resistance to gas exchange with the surrounding water is lower in submergence-acclimated than in non-acclimated plants. Such differences in diffusion resistance between the leaf types are ecologically relevant, because the levels of free CO_2 in for example the main stream of the river Rhine and its floodplains range from values of 15 to 90 µM (0.04 – 0.24 kPa; van den Brink et al. 1993), which is well in the range where acclimated leaves perform better than non-acclimated leaves (Fig. 3).

The cuticle of the lamina will be the major barrier for gas diffusion from the water column into the plant, as stomata are most likely not functioning under water and thus gases need to cross this waxy layer to enter the lamina. Recent work on several amphibious plant species showed that both cuticle thickness and cuticle resistance to oxygen diffusion were lower in leaves that were formed under water compared to aerial leaves of the same species (Frost-Christensen et al. 2003). It is therefore likely that the differences in gas diffusion resistance between the *Rumex* leaf types are the result from differences in cuticle thickness and resistance.

Summarising, this study modifies the dogma that internal oxygen concentrations always decrease to dramatically low levels when plants are submerged. It is still true that the internal oxygen pressure of petioles of *R. palustris* under water highly depends on the oxygen concentration of the water column, but if the water is not stagnant, oxygen pressures within both acclimated and non-acclimated shoots will not drop to the critical level that prevents aerobic metabolism. We also conclude that acclimation of the shoot to submergence is beneficial to gas exchange between the water column and the plant, since it involves a reduction of the diffusion resistance to gases. This is not only functional for increasing diffusion of oxygen from the water column into the plant, but also for increasing the influx of CO_2, which benefits under water photosynthesis.

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Chapter 5

Submergence-induced morphological, anatomical and biochemical responses in a terrestrial species: consequences for gas diffusion resistance and photosynthetic performance

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Abstract

Gas exchange between the plant and the environment is severely hampered when plants are submerged, leading to oxygen and energy deficits. A straightforward way to reduce these shortages of oxygen and carbohydrates would be continued photosynthesis under water, but this has received only little attention. Here, we combine several techniques to investigate the consequences of anatomical and biochemical responses of the terrestrial species *Rumex palustris* to submergence for different aspects of photosynthesis under water.

The orientation of the chloroplasts in submergence-acclimated leaves was towards the epidermis instead of the intercellular spaces, indicating that under water CO_2 diffuses through the cuticle and epidermis. Interestingly, both the cuticle and the epidermal cell wall thickness were significantly reduced upon submergence, suggesting a considerable decrease in diffusion resistance. This decrease in diffusion resistance greatly facilitated underwater photosynthesis, as indicated by higher underwater maximum photosynthesis rates, lower CO_2 compensation points and decreased photorespiration rates. Moreover, increased influx of CO_2 reduced excitation pressure of the electron transport system, thereby reducing the risk of photo damage. Furthermore, Rubisco contents were reduced in aquatic leaves, indicating a lower carboxylation capacity. Electron transport capacity was also reduced in these leaves, but not as strong as the reduction in Rubisco, indicating a

substantial increase of the ratio between electron transport and carboxylation capacity upon submergence. This novel finding is striking since this ratio is believed to be very conservative.

The increased CO_2 influx and altered photosynthesis biochemistry upon submergence greatly enhanced underwater photosynthesis and will increase the survival during submergence in this terrestrial species.

List of abbreviations

A_g	Gross assimilation rate
A_n	Net assimilation rate
C_i	CO_2 concentration in intercellular airspaces
D	Fraction of light energy that is dissipated in light
E	Fraction of excess energy
F_m	Maximum fluorescence in dark-adapted state
F_m'	Maximum fluorescence in light
F_v	Variable fluorescence in dark-adapted state
F_v'	Variable fluorescence in light
F_v/F_m	Maximum quantum yield of PSII, i.e. quantum yield in dark-adapted state
F_0	Minimal fluorescence in dark
F_0'	Minimal fluorescence in the light
J_C	Electron transport rate calculated from gas exchange measurements
J_F	Electron transport rate calculated from chlorophyll fluorescence
J_{max}	Electron transport capacity
LM	Light microscopy
L	Fraction of light energy that is dissipated in the dark, inherent inefficiency.
LMA	Leaf mass per unit leaf area (g m^{-2})
P	Fraction of light energy that is consumed by electron transport
PPFD	Photosynthetic Photon Flux Density
PSI	Photosystem I
PSII	Photosystem II
q_N	Non-photochemical quenching
q_P	Photochemical quenching
R_D	Respiration rate in dark
R_L	Respiration rate in light
R_P	Photorespiratory CO_2 release
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
V_O	Oxygenation rate
V_C	Carboxylation rate
V_{Cmax}	Carboxylation capacity
Γ*	CO_2 compensation point in the absence of R_L
$\Phi_{PSII} = \Delta F/F_m$'	Quantum yield of PSII electron transport

Introduction

Complete submergence severely inhibits gas exchange between the plant and the environment, because gas diffusion in water is approximately 10^4 slower than in air. This can lead to oxygen deficiency in the plant and concomitantly energy deficits due to hampered aerobic metabolism (Crawford & Brändle 1996). Well-known adaptations of plants to submergence include elongation of shoot organs to restore contact with the atmosphere (Voesenek et al. 2004) and the ability to switch to anaerobic metabolism to generate ATP in the absence of O_2 (Perata & Alpi 1993). A surprisingly poorly investigated phenomenon in submerged terrestrial plants to reduce the shortages of oxygen and carbohydrates is the potential for sustained photosynthesis under water (He et al. 1999; Vervuren et al. 1999).

The poor gas diffusion under water, however, also severely limits inorganic carbon supply for photosynthesis. Therefore, specialized aquatic plant species have developed CO_2 concentrating mechanisms (Bowes et al. 2002) or can use HCO_3^- (Maberly & Madsen 2002) or utilize sediment CO_2 (Pedersen et al. 1995) as a carbon source to overcome this limitation. As a result of the low free CO_2 availability in water, underwater photosynthesis in non-specialized plants is characterized by low maximum photosynthesis rates and low affinity for CO_2 due to high gas diffusion resistance (Maberly & Madsen 1998; Sand-Jensen & Frost-Christensen 1999; Madsen & Maberly 2003)

Recently, an oxygen micro electrode study on the flooding-tolerant species *Rumex palustris* showed that internal oxygen pressures are higher after acclimation to submergence (Mommer et al. 2004), suggesting reduced gas diffusion resistance in submerged acclimated leaves. This finding encouraged us to investigate the photosynthetic consequences of acclimation to submergence in this terrestrial species, which is a well-explored model species for terrestrial plant responses to submergence (Visser et al. 1996b; Cox et al. 2003; Voesenek et al. 2003b). Photosynthetic acclimation to submergence has hardly been investigated in terrestrial species, but more is known from work on amphibious plant species. These plants grow in the transition from land to water and develop both specialized terrestrial and aquatic leaves. Sand-Jensen et al. (1992) and Frost-Christensen & Sand-Jensen (1995) showed that aquatic leaves of these species have increased underwater CO_2 assimilation rates compared to their terrestrial counterparts, as a result of reduced gas diffusion resistance of the leaves. Frost-Christensen et al. (2003) also showed that this reduced gas diffusion resistance in aquatic leaves of amphibious plant species originates from reduced cuticle thickness and its reduced resistance for gases such as O_2. Photosynthesis rates may, however, not only be hampered by diffusional resistances (Long & Bernacchi 2003) but also by biochemical limitations (Centritto et al. 2003). Reduced Rubisco activities have been observed in aquatic leaves of amphibious species relative to their terrestrial leaves, and the

aquatic leaves, therefore, have a reduced carboxylation capacity (Farmer et al. 1986; Beer et al. 1991).

Here, we investigate if the terrestrial plant *Rumex palustris* shows responses upon submergence, such as a reduced cuticle thickness in submergence-acclimated leaves and consequently higher underwater photosynthesis rates and lower CO_2 compensation points that may be expected from the analogy with amphibious species. Furthermore, we explore novel responses of photosynthesis in response to submergence. An aspect which has not received much attention in amphibious plants species is that underwater the internal CO_2 concentration is relatively low compared to internal O_2 concentration, which favors oxygenation reactions of Rubisco over carboxylation (Ogren 1984). Underwater photosynthesis is, therefore, predicted to be characterized by high photorespiration rates (Lloyd et al. 1977; Salvucci & Bowes 1982). Here, we report the first *in vivo* estimates of photorespiration rates in higher plants under water. Another consequence of an aquatic environment is the relatively low availability of electron sinks in general (MacKenzie et al. 2004), which may impose relatively large excitation pressure on the electron transport system (Niyogi 2000). Increased internal CO_2 concentrations as result of acclimation to submergence might lower this pressure and thus reduce potential photoinhibition. Furthermore, we investigate if, next to reduced diffusion resistance, submergence acclimation also involves changes at the biochemical level of photosynthesis. If acclimation to submergence in terrestrial species resembles differences between the leaf types of amphibious species (Beer et al, 1991), we would expect reduced amounts of Rubisco protein in submergence-acclimated leaves of *R. palustris*. As different processes of the photosynthetic machinery are highly coordinated and kinetic properties are tuned to each other (von Caemmerer 2000), electron transport capacity was expected to show the same response to submergence as described for carboxylation capacity.

Materials and methods

Plant material and growth conditions

Rumex palustris Sm. seeds were germinated for 10 days in a petri dish on moistened filter paper at temperatures of 22°C during daytime (Photosynthetic Photon Flux Density (PPFD) 20 µmol m^{-2} s^{-1}) and 10°C at night. The seedlings were transplanted to pots of 0.3 L, containing a sieved sand/potting soil mixture (1:1 v:v) and grown for another 3 weeks in a growth chamber at a PPFD 200 µmol m^{-2} s^{-1} (SON-T plus 600 W and TLD Reflex 36W/840R, Philips, Eindhoven, the Netherlands; day/night cycle 16h/8h; temperature 20°C). The plants were watered once a week with ¼ Hoagland's nutrient solution. To investigate the effect of submergence on photosynthetic characteristics, one group of plants was completely

submerged, whereas the other group was kept drained. The submerged plants, hereafter referred to as plants with 'aquatic leaves', were flooded in basins filled with tap water, which was circulated with a flow rate of 3 L min^{-1}. Plants of the drained treatment (having 'terrestrial leaves') were placed in a similar basin as described above, but without being filled with water. In both treatments, PPFD was 200 µmol m^{-2} s^{-1}, the day/night cycle was 16/8 h, and the temperature 20°C. The treatments lasted 10 days, and the plants developed at least two new leaves during the treatments. The apical halves of these new leaves were used in all analyses.

Leaf anatomy and morphology

Leaf anatomy was investigated with light microscopy (LM) and transmission electron microscopy (TEM). Samples were taken from the top of the leaf and fixed in 2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 for 2h at room temperature, followed by post-fixation in 1% (w/v) osmium tetroxide in the same buffer for 1h. The tissue was dehydrated through an ethanol series with steps of 10% and via propylene oxide embedded in Spurr's resin (Spurr 1969). For light microscopy, sections of 1µm were stained with toludine blue (0.1% in 1% borax) and viewed with Leitz Ortoplan microscope (Wetzlar, Germany). For TEM, thinner sections (20 nm) were post-stained with uranyl acetate and lead citrate according to standard procedures and viewed with a transmission electron microscope (JEOL JEM 100CX II, Jeol, Tokyo, Japan). Thicknesses of the leaves (4 counts within sample, n=3), outer cell walls of epidermis cells and cuticles (20 counts within sample, n=2) were measured with digital software.

Cryo-field emission scanning electron microscopy (SEM) techniques were used for determination of 3D dimensions the leaf surface. Leaf discs were glued onto a stub with colloidal carbon adhesive and frozen in slushy liquid N_2. The samples were transferred in a transfer holder under vacuum into a cryo-preparation chamber of -180°C (Oxford Alto 2500, Gatan Inc., Oxford, UK). Samples were then freeze-dried for 4 min -90°C and sputter-coated with 4 nm gold palladium and conveyed under high vacuum to the cold stage of a scanning electron microscope (FESEM-JSM 6330, Jeol, Tokyo, Japan).

Other shoot parameters measured included leaf length and width and leaf mass per area (LMA). LMA was calculated from leaf area (Li-3000, Li-Cor Inc., Lincoln, NE, USA) and dry mass (determined after drying for 48h at 80°C) of the lamina at which measurements were conducted. Non-structural carbohydrate-free LMA was calculated by subtraction of the mean weight contribution of non-structural carbohydrates from the overall LMA replicates.

Biochemical analyses

Chlorophyll content was determined spectrophotometrically (UV1250 Shimadzu corporation, Maryland, Columbia, USA) after extraction of the chlorophyll pigments with dimethylformamide for 7 d in dark at 4°C in samples of the leaves on which photosynthetic measurements were performed. Equations of Inskeep & Bloom (1985) were used to calculate the chlorophyll a and b pigment concentrations.

Determination of carbohydrate content of the leaves was based on assay of Colmer et al. (2001) and performed on a separate, but identically grown set of plants. Leaves were harvested between 9.00 and 10.00 am, quickly frozen in N_2 and stored in a freezer at -80°C until further processing. After freeze-drying 10 mg of fine cut material was used to measure non-structural carbohydrates. All soluble sugars (hexose units) were extracted in 4 ml ethanol (80%) during 30 min in a shaking water bath at 80°C and this step was repeated with 2 ml ethanol after centrifugation. Insoluble sugars (starch) were analyzed by boiling the residue in 3.5 ml demineralized water for 3 h and then incubating the solution for 24 h at 37 °C with 1 ml Na^+ acetate buffer containing amyloglucosidase (75 U mg^{-1}, EC 3.2.1.3), pH 4.5. Both soluble sugars in the ethanol extraction and the glucose released from the hydrolysis of starch were measured with a Shimadzu UV1250 spectrophotometer (Shimadzu Corporation, Columbia, Maryland, USA) using anthrone as a color reagent (Yem & Willis 1954).

For Rubisco protein analyses, 28 days instead of 42 days old plants were used, because reduced extraction efficiency was observed in the older plants, possibly due to high amounts of secondary metabolites. Aerial photosynthesis characteristics on just fully developed leaves of these plants were performed as described below. Frozen samples of approx. 6 cm^2 were ground in eppendorf tubes in an extraction buffer containing 200 mM Tris (pH 7.8), 20 mM $MgCl_2$, 150 µM $NaCO_3$, 20% glycerol, 1mM EDTA, 10 µM DTT, 0.5 % Triton X-100, 8 mM amino-n-caproic acid, 1.6 mM benzamidine and 3% w/v PVPP. Samples were centrifuged at 14000 g for 10 min at 4°C and the pellet was discarded. Salt solutions were added to the supernatant to create a final concentration of 10 mM $NaHCO_3$ and 20 mM $MgCl_2$. Protein samples were run on SDS-PAGE gels (17%) for 4 h at 100 V (Westbeek et al. 1999). Five BSA samples with different concentrations were also loaded on the gels, serving as a calibration curve. After staining the gels with Coomassie (brilliant) blue for 30 minutes and subsequently destaining overnight, gels were scanned using custom built image analysis. Rubisco content was calculated from the large subunit (molecular mass of 55 kDa). Total soluble protein content was measured using the Bradford reagens (Bradford 1976).

Gas exchange measurements in air and under water

CO_2 assimilation rates in air (n=6) were measured on ‘terrestrial’ and ‘aquatic’ leaves with an open gas exchange measurement system where air was led through leaf chambers.

Differences in CO_2 and H_2O partial pressure between air flows entering and leaving the leaf chamber were measured with an infra red gas analyzer (Li-Cor 6262, Lincoln, Nebraska, USA). The leaf chambers contained a window (67 x 69 mm) through which the light beam of a lamp mounted in a slide projector was transmitted (see for details (Pons & Welschen 2002). PPFD at leaf level was 1000 µmol m^{-2} s^{-1}, which was enough to saturate photosynthesis; leaf temperature 20°C; vapor pressure difference between leaf and air was between 0.4 and 0.5 kPa. Submerged plants were taken out of the water 1h before the measurement started and placed in a plastic bag to maintain turgor of the leaves. Only the measured part of the leaf was released from the bag during the measurement. This treatment did not result in water deficit.

Under water gas exchange was measured as oxygen evolution in water with different free CO_2 concentrations (n=6). Oxygen release and uptake of squared leaf parts (1cm^2) were recorded using a Clark type oxygen electrode positioned at the bottom of a cuvette (Chlorolab 1, Hansatech Instruments, Norfolk, UK; Delieu & Walker 1972). The cuvette was filled with 1.5 ml 50 mM MES-BPT buffer (pH 6.5) containing 5 mM$CaCl_2$, 1.5mMKCl, 1 mM NaCl and $NaHCO_3$ concentrations ranging from 23 µM to 23 mM resulting in a free CO_2 concentration of 10 µM to 10 mM respectively. PPFD under water was 400 µmol m^{-2} s^{-1}. The leaf parts were fixed in the cuvette so that light distribution was homogeneous. Oxygen concentrations were kept between 110 and 170 µM (40-60% of air-saturation) to limit variation in diffusion gradients between the leaf parts and the buffer. A magnet, driven by a custom stirrer unit provided circulation of the buffer in the cuvette, thereby minimizing boundary layers. A cylinder around the cuvette that contained circulating thermostated water using a thermostatic water bath (Haake DC 50, Karlsruhe, Germany) maintained the water temperature at 20°C.

Measurement of chlorophyll fluorescence in combination with gas exchange

Photorespiration rates were estimated in terrestrial and aquatic leaves by combining gas exchange measurements with chlorophyll *a* fluorescence measurements. The measurements in air were performed with the aerial gas exchange system as described above, except that modified Parkinson cuvettes were used (ø18mm; PP systems, Hitchin, UK; Pons & Welschen 2002). Holders for the fibre optics of the a portable PAM2000 chlorophyll fluorometer (Walz GmbH, Effeltrich, Germany) were placed on these chambers, without shading the leaf (Pons & Welschen 2003). Measurements were performed at two CO_2 concentrations (ambient and saturating, 37 and 170 Pa CO_2 respectively) and two O_2 concentrations (ambient and photorespiration depressing, 21 and 1 kPa O_2; n=6). Dark respiration rates (R_D) and maximum quantum yield of photosystem II (F_v/F_m) were measured after 30 min in the dark. The saturating pulse and measuring light were maximal for

determining maximum fluorescence (F_m) and revealed appropriate signals, also for minimal fluorescence (F_0). Photosynthesis rate was recorded after 30 min in saturating light (PPFD 1000 μmol m^{-2} s^{-1}), and accompanying quantum yield measurements ($\Phi_{PSII} = \Delta F/F_m'$) were replicated three times with 5 min intervals and averaged for each CO_2 concentration. Minimum fluorescence yield after induction (F_0') was determined after a far-red light pulse following immediately after the light was switched off.

After these aerial measurements the plants were returned to their respective treatment basin overnight. Underwater gas exchange and fluorescence measurements were performed on the same leaves the next morning.

For these underwater measurements the fluorescence fibre probe was inserted in the water cylinder of the Chlorolab1 cuvette, under such an angle that the light beam of the projector was not impeded. The measurements under water were again performed on leaf parts (1 cm^2), following the same protocol as described above. CO_2 concentrations were 250 and 2000 μM, which have been shown before to be distinctive for the CO_2 response of the leaf types (Mommer et al. 2004). PPFD under water was 400 μmol m^{-2} s^{-1} since higher PPFD resulted in inaccurate Φ_{PSII} at the lowest CO_2 concentrations. R_D measurements were performed at an oxygen concentration of 230 μM (80% of air-saturation), which does not limits respiration in *Rumex* species (Laan et al. 1990).

Calculations and assumptions

Electron transport rate was calculated from aerial gas exchange measurements (J_C) (von Caemmerer 2000):

$$J_C = \frac{4\left(A_n + R_L\right)\left(C_i + 2\Gamma^*\right)}{C_i - \Gamma^*} \qquad (1)$$

where A_n (net assimilation rate) and R_L (respiration rate in light assumed to equal R_D) were recorded directly from aerial gas exchange measurements; C_i represents CO_2 concentration in intercellular spaces and Γ* the CO_2 compensation point in the absence of R_L. Γ* was assumed to be 3.06 Pa CO_2 in normal air at 101.3 kPa atmospheric pressure, as observed in *Nicotiana tabacum* (von Caemmerer et al. 1994). The factor 4 indicates the stoichiometry between transported electrons and assimilated CO_2.

We measured significantly higher transpiration rates in aquatic than in terrestrial leaves in the aerial gas exchange measurements (data not shown), probably because of cuticular transpiration in the aquatic leaves in air. As a result, C_i was not measured accurately in these leaves in air, since the basic assumption that gas and water transport via the cuticle is negligible may not have been true (von Caemmerer & Farquhar 1981). Still, in the calculations for electron transport rate (J_C) in aquatic leaves we had to use C_i as a

parameter. Since we expected the measured C_i in aquatic leaves to be overestimated, we used the mean value of C_i in the terrestrial leaves as the best possible estimate for C_i in the aquatic leaves.

Electron transport measurements can, alternatively, be obtained from fluorescence analyses (J_F) following (Genty et al. 1989).

$$J_F = 0.5 * abs * PPFD * \phi_{PSII} \qquad (2)$$

where 0.5 is a factor that accounts for partitioning of energy between PSI and II and leaf absorptance (abs) was calculated from chlorophyll concentrations (Table 2) according to Evans & Poorter (2001). This resulted into absorption coefficients of 0.79 and 0.76 for terrestrial and aquatic leaves respectively.

J_F was calibrated with measurements of J_C in the aerial gas exchange system under low O_2 conditions (1kPa) so that oxygenation and photorespiration were suppressed. The terrestrial and aquatic leaves were calibrated at different CO_2 concentrations (38 and 170 Pa CO_2 respectively), in order to have comparable A_n in both leaf types. For calibration, photorespiration was suppressed by lowering the oxygen pressure from 21 to 1kPa O_2, and Γ* was than estimated as 1/21 part of 3.06 Pa CO_2. Mean values of the J_C/J_F ratio ± SE were 0.68±0.03 and 0.78±0.04 for the terrestrial and aquatic leaves respectively. We used for each replicate the corresponding calibration factor. The use of this calibration factor assumes an equal proportion of other electron sinks at different O_2 and CO_2 concentrations in air and water and constant optical properties. The difference between gross photosynthesis rate ($A_g = A_n+R_L$) and the electron transport rate (J_C) was used as an estimate of electron transport involved in oxygenation (V_O) and consequent photorespiratory CO_2 release (R_P).

In the younger leaves of *R. palustris* electron transport capacity (J_{max}) was calculated from aerial assimilation rates at saturating light intensities at 170 Pa CO_2 (see equation 1 and von Caemmerer 2000). As explained before, C_i of the aquatic leaves was assumed to be similar to C_i in the terrestrial leaves, which might have resulted in an underestimation of J_{max} in the first. To show that terrestrial leaves have an entirely normal ratio of electron transport capacity (J_{max}) over carboxylation capacity (V_{Cmax}), this latter parameter was estimated from the slope of the aerial CO_2 response curves under saturating light according to Farquhar & von Caemmerer (1982).

Photochemical (q_P) and non-photochemical (q_N) quenching components were calculated from chlorophyll fluorescence measurements (*cf* van Kooten & Snel 1990). Partitioning of absorbed photons in photosystem II was calculated by dividing total absorbed energy over inherent inefficiency in dark ($L=1- F_v/F_m$), thermal dissipation ($D=F_v/F_m-F_v'/F_m'$), photosynthetic electron transport ($P=\Delta F/Fm'$) and excess energy ($E=1-L-D-P$) (Demmig-Adams et al. 1996; Kato et al. 2003).

Differences between leaf types were analyzed with Student's t-tests. Levene's test was used to check the homogeneity of variances. Ln-transformation was applied if deviation from homogeneity of variance was found.

Results

Leaf morphology and anatomy change as a result of submergence

Leaf morphological and anatomical features of *Rumex palustris* showed strong plasticity in response to submergence. The leaves developed during submergence (referred to as 'aquatic leaves') were elongated and thin in comparison with 'terrestrial leaves' developed completely in air (Table 1, Fig. 1a,b). Furthermore, the epidermal cell wall and cuticle thickness were greatly reduced in aquatic leaves compared to terrestrial leaves (Fig. 2).

Leaf mass per area (LMA) was considerably lower in aquatic leaves compared to terrestrial leaves (Table 1). This difference was largely caused by starch accumulation in the terrestrial leaves (Table 1). Soluble sugar contents were not significantly different between the leaf types. When LMA was corrected for these non-structural carbohydrates, the difference between the leaf types was strongly reduced, but a significant difference still remained (Table 1).

Table 1. Morphological and anatomical characteristics of terrestrial and aquatic leaves of *R. palustris*. LMA is leaf mass per area; NSC-free LMA is leaf mass per area minus the weight contribution of non-structural carbohydrates (NSC); #stomata is number of stomata per unit leaf area; #epidermis number of epidermis cells per unit leaf area. Values are means ± SE. Data were analyzed with Student's t-tests, and ln-transformation was applied if discrepancy from homogeneity of variance were found. **P<0.01, ***P<0.001, n.s. not significant.

Parameter		***Terrestrial leaves***	***Aquatic leaves***	***n***	
Leaf length (cm)		9.0±0.1	16.7±0.7	6	***
Leaf width (cm)		2.7±0.0	2.0±0.1	6	***
Leaf area (cm^2)		20.1±0.8	14.4±0.7	10	***
Leaf thickness (µm)		222±4	178±8	3	**
LMA ($g\ m^{-2}$)		47.8±1.46	20.0±0.7	10	***
Starch ($mg\ g^{-1}DW$)		415.7±29.7	31.6±6.0	8	***
Sucrose ($mg\ g^{-1}DW$)		30.2±2.9	24.8±1.4	8	n.s.
NSC-free LMA ($g\ m^{-2}$)		26.5±0.8	18.9±0.7	8	***
#stomata (mm^{-2})	adaxial	29±2	73±6	5	***
	abaxial	47±5	85±6	5	**
#epidermis cells (mm^{-2})	adaxial	224±24	923±33	5	***
	abaxial	271±23	943±28	5	***

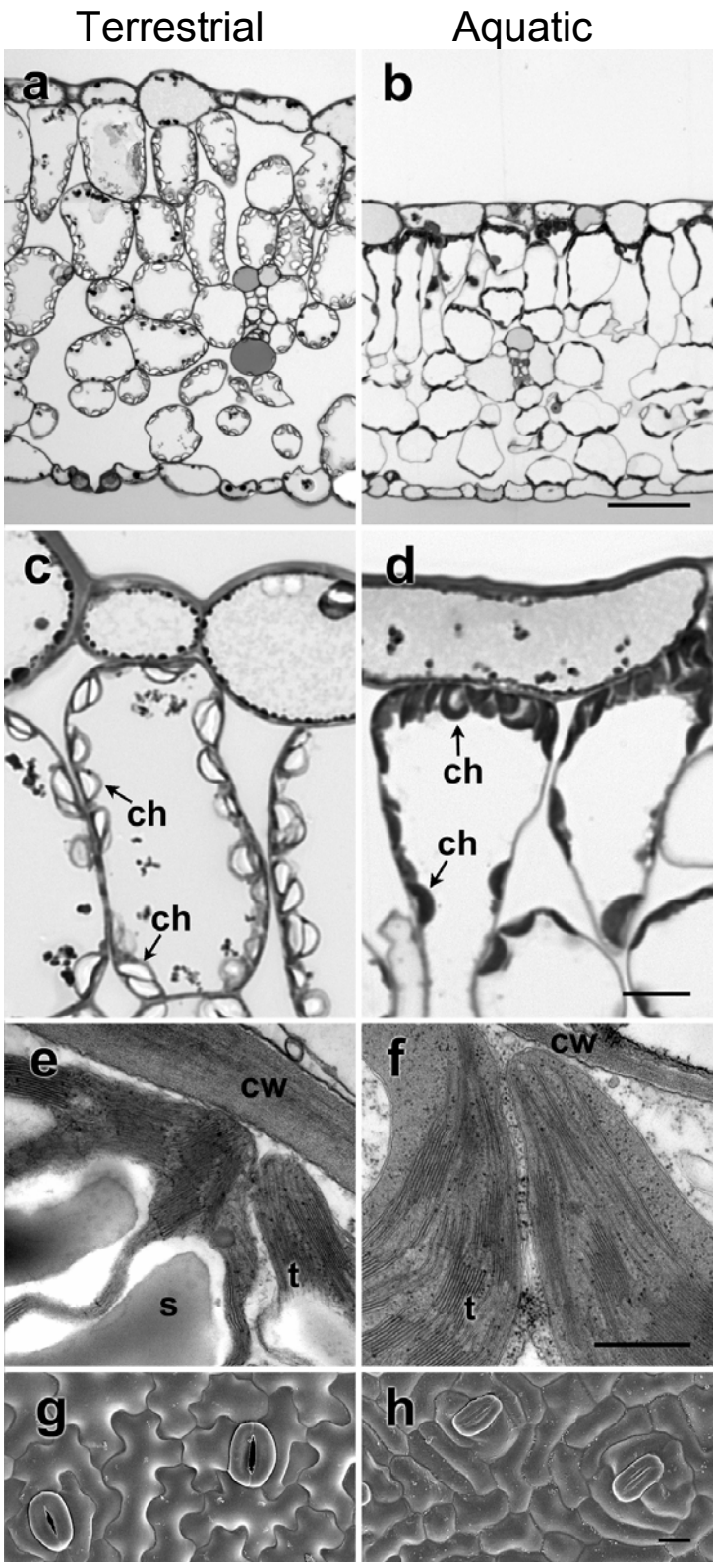

Figure 1. Anatomy of terrestrial and aquatic leaves of *R. palustris*. LM image of a transverse leaf section of (a) terrestrial and (b) aquatic leaf (250 x; scale bar represents 50 µm). Detail of the adaxial epidermis and mesophyll cells showing the position of chloroplasts (chl) in (c) terrestrial and (d) aquatic leaf (1000x; scale bar represents 10 µm). TEM image of chloroplast with thylakoïd structure (t). (e)Terrestrial leaf showing starch deposition (s) and (f) aquatic leaf (11,000 x, scale bar represents 500 nm). SEM image of leaf surface showing stomata and epidermis cells of (g) terrestrial and (h) aquatic leaf (300 x, scale bar represents 20 µm).

In contrast to aquatic leaves of many amphibious plants, the aquatic leaves of *R. palustris* contained stomata, even more than their terrestrial counterparts (Table 1, Fig. 1g,h). The pavement cells in the epidermis of terrestrial leaves were clearly 'jigsaw'-shaped, whereas the epidermal cells of the aquatic leaves were more rectangular (Fig. 1 g,h), as has been

observed in elongated organs (Glover 2000). The pavement cells of the elongated aquatic leaves were smaller than those of the terrestrial leaves, as shown by the larger number of cells per mm^2 and per leaf lamina in aquatic leaves (Table 1). The chloroplasts in the aquatic leaves were clearly orientated towards the epidermis instead of the intercellular orientation in terrestrial leaves (Fig. 1c,d).

Acclimation to submergence leads to higher underwater CO_2 assimilation rates

In order to investigate the functional consequences of these morphological and anatomical acclimation responses, underwater oxygen evolution was measured in both leaf types. Aquatic leaves already had a net positive assimilation rate (A_n) under water at 10 µM free CO_2 (equilibrium pressure at 25 Pa CO_2, which is slightly below atmospheric equilibrium), whereas in the terrestrial leaves the minimal CO_2 concentration for positive A_n was between 100 and 250 µM CO_2 (Fig. 3a). Thus aquatic leaves had much lower underwater CO_2 compensation points than terrestrial leaves. The slope of the underwater CO_2 response curves was steeper in aquatic leaves than in terrestrial ones (Fig. 3a), indicating a higher affinity for CO_2. Maximum assimilation rates under water were also higher in aquatic leaves

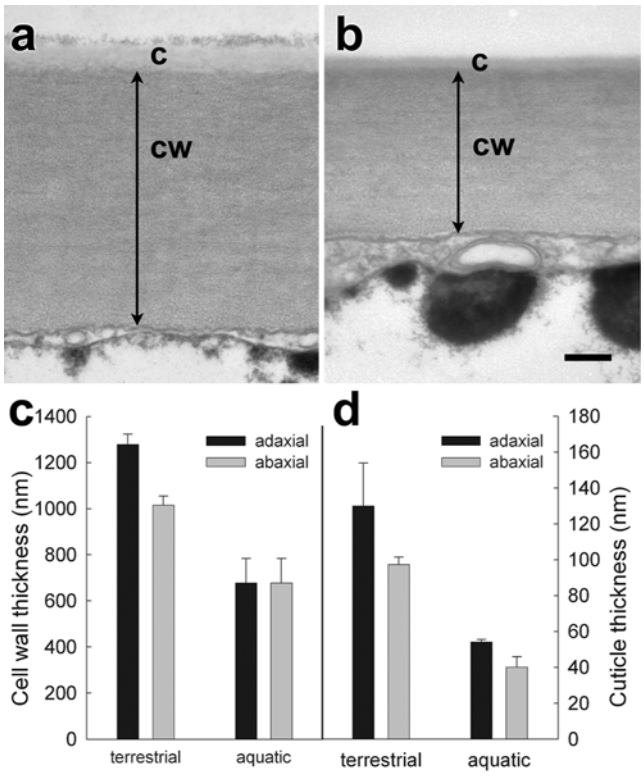

Figure 2. Cell wall and cuticle thickness of epidermis cells of terrestrial and aquatic leaves of *R. palustris*. TEM image of a transverse leaf section showing the adaxial epidermal cell wall (cw) with a cuticle (c). (a) Terrestrial leaf and (b) Aquatic leaf (36,300x, scale bar represents 200 nm). Graphs show the difference in thickness of (c) cell wall and (d) cuticle. Data are means ± SE.

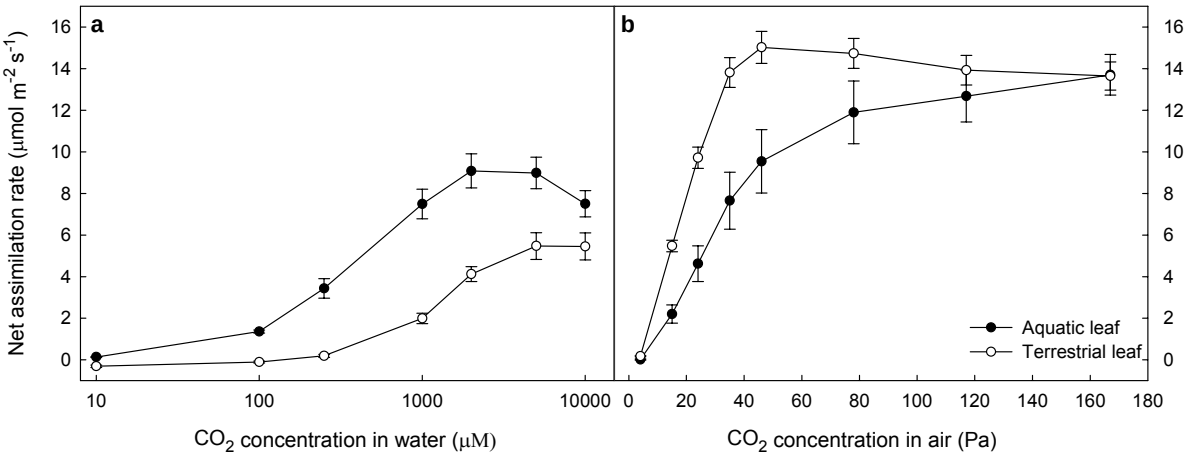

Figure 3a,b. CO_2 response curves of terrestrial and aquatic leaves of *R. palustris* under water (a, in O_2 production) and in air (b, in CO_2 production) at PPFDs of 400 and 1000 µmol m^{-2} s^{-1} respectively. CO_2 concentrations in the water (a) and partial pressure (b) are plotted on the x-axes. Data are means ± SE, n=6.

than in terrestrial leaves, even when CO_2 concentrations in the water were increased to 2000 µM.

Aerial gas exchange measurements also revealed differences between the two leaf types in response to CO_2 (Fig. 3b) but these were opposite to those under water (Fig. 3a), since A_n was lower in aquatic leaves compared to terrestrial leaves at ambient CO_2 concentrations in air (Fig. 3b).

In terrestrial leaves net assimilation rates (A_n) were much lower in water than in air, indicating that CO_2 is limiting photosynthesis under water, even at the extremely high CO_2 concentration of 2000 µM (Fig. 4a,b). The highest CO_2 concentrations both in water and air yielded similar A_n in aquatic leaves (Fig. 4a,b), but electron transport rates (J_F) were higher in air than in water in these leaves, which might be explained by a difference in PPFD between the measurements in air and water. A_n values in air were different between batches of plants (Fig. 3b, 4b, Table 2), in particular for aquatic leaves, which might be explained by variation in stomatal control after de-submergence. Still, the relative responses to submergence were identical.

The underwater gas exchange measurements together point to a greater CO_2 influx as a result of the significant decrease in diffusion resistance in response to submergence, which is indicated by reduced CO_2 compensation points and higher A_n under water in the aquatic leaves.

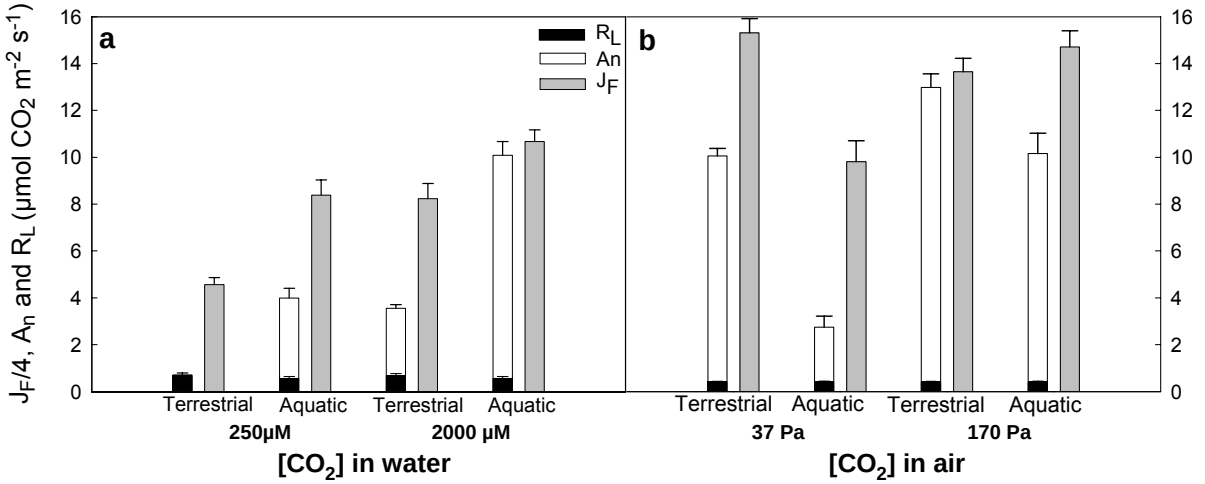

Figure 4a,b. Electron transport rate (J_F) measured with chlorophyll fluorescence (calibrated with gas exchange measurements at 1% O_2) and photosynthesis rate (A_n) and respiration (R_L) (stacked bar) measured with gas exchange in terrestrial and aquatic leaves of *R. palustris* under water (a) and in air (b). PPFD was 400 and 1000 µmol m^{-2} s^{-1} for the measurements under water and in air respectively. The difference between J_F and A_n+R_L reflects the estimate of photorespiration. Data are means ± SE, n=6.

Acclimation to submergence results in decreased photorespiration rates under water

In order to investigate if the reduced diffusion resistance in aquatic leaves affected photorespiration rates, we determined the difference between photosynthetic electron transport rate (J_F) measured by means of chlorophyll fluorescence and gross assimilation rate (A_g=A_n+R_L), based on gas exchange. This difference is considered to be representative for *in vivo* photorespiration rates. Terrestrial leaves had substantial J_F under water at 250 µM CO_2, but no net CO_2 assimilation (Fig. 4a). At this CO_2 concentration, which is already 15 times higher than in air-saturated water, the terrestrial leaf was apparently transporting electrons in a closed circuit, assuming that no other quanitatively important electron acceptors are active. The electron transport rate involved in carboxylation (V_C) was apparently compensated by an equally large photorespiration rate (R_P) plus a small share of dark respiration rate (R_L).

Both J_F and A_g increased considerably in both leaf types by increasing the CO_2 concentration in the water from 250 to 2000 µM (Fig. 4a), indicating that the system under water was limited by electron acceptors at low CO_2 concentration. There was a clear net O_2 production at 2000 µM in terrestrial leaves, and J_F was also higher than at the low CO_2 concentration, but photorespiration rates were still substantial in these leaves as indicated by the difference between J_F and A_g (Fig. 4a). At this high CO_2 concentration J_F and A_g were, however, identical in the aquatic leaves, indicating that photorespiration in these leaves was so low, that it could not be detected anymore.

Table 2. Chlorophyll fluorescence parameters of terrestrial and aquatic leaves of *R. palustris*, measured under water and in air at different CO_2 concentrations. Under water, PPFD was 400 µmol m^{-2} s^{-1} and O_2 concentration 110-170 µM (40-60% of air-saturation). In air, PPFD was 1000 µmol m^{-2} s^{-1} and O_2 concentration 21 kPa. F_v/F_m is the maximum quantum efficiency of PSII; q_P is photochemical and q_N is non-photochemical quenching component. Values are means ± SE, n=6. Data were analyzed with Student's t-tests. * P<0.05, ***P<0.001, n.s. not significant.

Measurement in	*Parameter*	*Terrestrial leaves*	*Aquatic leaves*	
Water	F_v/F_m	0.795±0.013	0.754±0.014	*
250 µM CO_2	q_P	0.381±0.022	0.597±0.032	***
	q_N	0.840±0.015	0.785±0.014	*
2000 µM CO_2	q_P	0.615±0.021	0.634±0.013	n.s.
	q_N	0.812±0.011	0.663±0.015	***
Air	F_v/F_m	0.821±0.002	0. 798±0.005	***
37 Pa CO_2	q_P	0.796±0.009	0.661±0.022	***
	q_N	0.488±0.042	0.752±0.025	***
170 Pa CO_2	q_P	0.789±0.010	0.766±0.008	n.s.
	q_N	0.620±0.027	0.592±0.012	n.s.

Interestingly, measurements on the two leaf types in air revealed patterns opposite to those found in underwater measurements. The difference between A_g and J_F in terrestrial leaves measured in air at 37 Pa CO_2 was 29%, indicating substantial photorespiration at ambient conditions (Fig. 4b). This difference disappeared when the CO_2 concentration was increased to 170 Pa. Photosynthesis was low in aquatic leaves when measured in air at 37 Pa CO_2, as indicated by low A_g and J_F as compared to terrestrial leaves. Moreover, the difference between these parameters was large (Fig. 4b), indicating high photorespiration in the aquatic leaves in air. A_g increased much more than J_F in these aquatic leaves at high CO_2 concentration and photorespiration consequently decreased but was still evident. Summarising, aquatic leaves had higher net underwater CO_2 assimilation rates combined with lower photorespiration rates than terrestrial leaves, when measured under water.

Aquatic leaves have lower non-photochemical quenching under water

Chlorophyll fluorescence measurements showed the excitation pressure on the electron transport chain and thus the risk of photodamage. The partitioning of absorbed photons was calculated over inherent inefficiency (L), photosynthetic electron transport (P), heat dissipation (D) and to processes which may potentially damage the plant (excitation pressure or excess energy, E). This partitioning depends on environmental cues such as light and

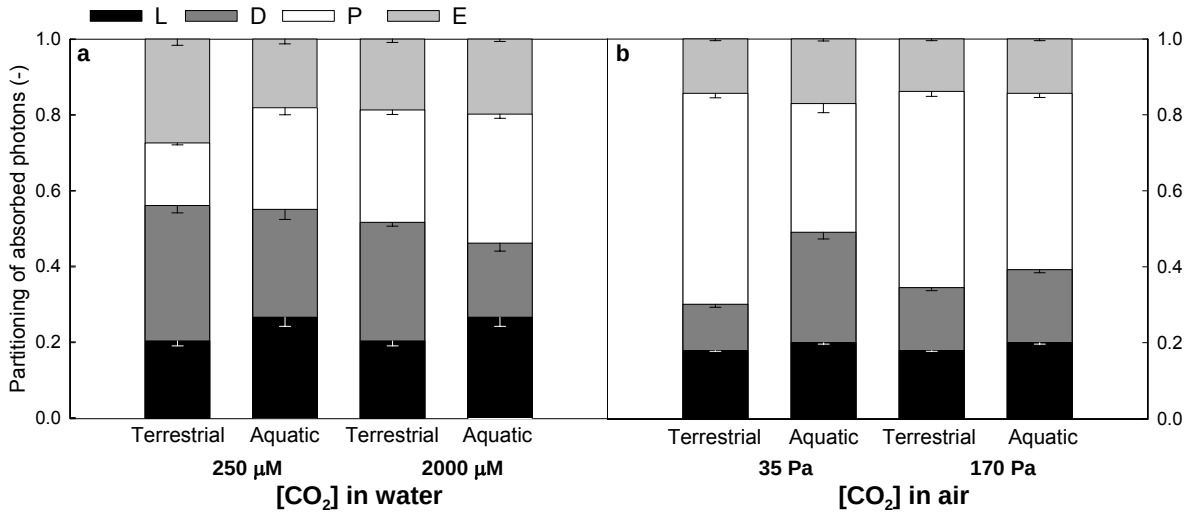

Figure 5a,b. Partitioning diagrams of total absorbed photons of terrestrial and aquatic leaves of *R. palustris* at different CO_2 concentrations under water (a) and in air (b). Partitioning parameters are inherent inefficiency in dark (L), thermal dissipation (D), electron transport (P) and excess energy (E). PPFD was 400 and 1000 µmol m^{-2} s^{-1} for the measurements in water and air respectively. Data are means ± SE, n=6.

temperature, but also on the availability of electron sinks, such as CO_2 and O_2 (MacKenzie et al. 2004). The latter was different in terrestrial and aquatic leaves because of their differences in diffusion resistance. In general, the leaf type with treatment combinations with the highest J_F, also showed the highest photochemical quenching (q_P) and thus also the highest partitioning to photosynthetic electron transport (P). The effect of the increased availability of electron sinks on partitioning was illustrated by the increased q_P in both leaf types with increasing CO_2 concentrations. Non-photochemical quenching (q_N) including proportions of absorbed photons directed to heat dissipation (D) and excess energy (E) were, as expected, highest in the situations with the lowest CO_2 availability; i.e. the terrestrial leaf at low CO_2 had the largest E fraction among the measurements in water and the aquatic leaf at low CO_2 had the largest E fraction among the measurements in air (Table 3, Fig. 5a,b). Furthermore, we observed a slight reduction of F_v/F_m when leaves where measured in water compared to air, and this decrease was stronger for aquatic leaves than for terrestrial leaves (Table 3), indicating some downregulation of PSII efficiency when PPFD exceeds 200 µmol m^{-2} s^{-1} under water.

The fact that the aquatic leaves showed higher photochemical quenching and lower non-photochemical quenching under water than did the terrestrial leaves indicates that acclimation to submergence reduced excitation pressure on the photosystems underwater (E) and therefore the risk of photodamage.

Table 3. Biochemical and photosynthetic characteristics in terrestrial and aquatic leaves of *R. palustris*. The third, just fully expanded leaf of *R. palustris* plants grown for 28 instead of the normal 42 days has been used for these analyses. J_{max} is electron transport capacity, i.e. electron transport rate under saturating light and is based on aerial gas exchange measurements and calculated following equation 1, under the assumption that C_i of aquatic leaves is equal to that of terrestrial leaves. PPFD was 1000 µmol m^{-2} s^{-1}. V_{Cmax} is calculated according to Farquhar & von Caemmerer (1982). Values are means ± SE, n=6. Data were analyzed with Student's t-tests, and J_{max}/Rubisco data were ln-transformed to obtain homogeneity of variance. **P<0.01, ***P<0.001.

Parameter	***Terrestrial leaves***	***Aquatic leaves***	
Chlorophyll (µmol m^{-2})	288.3±16.7	211.7±8.9	**
Soluble protein (mg m^{-2})	4.3±0.2	2.2±0.2	***
Rubisco protein (µmol m^{-2})	3.03±0.17	0.75±0.07	***
J_{max}/V_{Cmax}	1.44±0.01	Not determined	
J_{max} (µmol e^- m^{-2} s^{-1})	119.6±4.6	63.3±3.0	***
J_{max}/Rubisco (s^{-1})	40.1±2.9	88.2±8.3	***

Aquatic leaves have a higher electron transport capacity relative to carboxylation capacity

According to the model described for C_3 photosynthesis (Farquhar & von Caemmerer 1982), carboxylation capacity (V_{Cmax}) can be derived from the slope of the aerial CO_2 response curve. However, the reduced slope of the aerial CO_2 response curve in aquatic leaves of *R. palustris* (Fig. 3b) may reflect not only a biochemical limitation in these leaves, but also diffusional limitation at the lower CO_2 concentrations. The uncertainty in calculating C_i in aquatic leaves precludes the calculation of the carboxylation capacity from gas exchange measurements. An alternative way to investigate if carboxylation capacity was reduced upon submergence is to measure the amount of Rubisco protein, which reflects *in vivo* Rubisco activity and thus V_{Cmax} at saturating light intensities (Woodrow & Berry 1988) when Rubisco is fully activated.

The Rubisco analyses showed that aquatic leaves of *R. palustris* contained 3 times less Rubisco protein on a leaf area and chlorophyll basis than terrestrial leaves (Table 2).

Since photosynthetic reactions are generally highly coordinated, with carboxylation capacity and electron transport capacity tuned to each other, we expected the reduced Rubisco in aquatic leaves to be accompanied by a comparable reduction of the electron transport capacity (J_{max}). J_{max}, estimated from gas exchange parameters at saturating light and CO_2 concentration was reduced in aquatic leaves compared to terrestrial leaves of these younger plants (Table 2). However, the reduction of J_{max} was much less than the reduction in Rubisco content. Consequently, the higher J_{max}/Rubisco ratio in the aquatic leaves (Table 2) suggests that submergence-acclimated leaves have a considerably higher photosynthetic

electron transport capacity relative to carboxylation capacity compared to non-acclimated leaves.

Discussion

Leaves of the terrestrial plant species *Rumex palustris* show pronounced morphological, anatomical and biochemical acclimation to submerged conditions. This resulted in differences in photosynthetic performance under water between the terrestrial and aquatic leaves. The underwater photosynthesis measurements show the immense capacity of this flooding tolerant species to adjust its morphology and physiology to submerged conditions.

Decreased diffusion resistance is beneficial for underwater CO_2 assimilation

The functionality of the response to submergence was evident from the higher maximum underwater photosynthesis rates in aquatic leaves than in terrestrial leaves of *R. palustris* (Fig. 3a). Another benefit of acclimation to submergence is the lowering of CO_2 compensation points in aquatic compared to terrestrial leaves of *R. palustris* (Fig. 3a), reaching values (<10µM) that are ecologically relevant and comparable to those of aquatic leaves of amphibious plants (Sand-Jensen & Frost-Christensen 1999). Affinity for CO_2 under water was higher in aquatic than in terrestrial leaves, as indicated by a steeper slope of the CO_2 response curve of the aquatic leaves (Fig. 3a), which is commonly observed in amphibious plants (Maberly & Spence 1989; Sand-Jensen & Frost-Christensen 1999). These differences in underwater gas exchange characteristics are clearly the result of a decreased diffusion resistance in response to submergence in this terrestrial species. This is most obvious from the underwater photosynthetic performance at low CO_2, since affinity for CO_2 is the combined result of all diffusional resistances over the leaf, although it can also be affected by biochemical limitations (Centritto et al. 2003). Under water, an important factor determining CO_2 affinity is considered to be cuticle resistance (Frost-Christensen et al. 2003). Cuticle resistance is determined by its thickness and the chemical composition of waxes and cutin (Lequeu et al. 2003). Cuticle thickness *per se* is not necessarily associated with resistance for water transport and gas diffusion, since no clear correlations between these parameters have been observed in several drought tolerant species (Schreiber & Riederer 1996; Kerstiens 1996a; Kerstiens 1996b). However, Frost-Christensen et al. (2003) showed that aquatic leaves of five amphibious species had a reduced cuticle thickness compared to terrestrial leaves and an accompanying reduced diffusional resistance for gases. We, therefore, conclude that it is very likely that the difference in underwater CO_2 affinity between the leaf types of *R. palustris* (Fig. 4a) will mainly be explained by a difference in cuticle thickness (Fig. 2a,b,d) and its putative decrease in resistance (Frost-Christensen et

al. 2003). The differences in CO_2 affinity between the leaf types were not related to boundary layer effects, since boundary layers were minimal and similar in the leaf discs in the well-stirred cuvette. It is not likely that stomata will contribute significantly to CO_2 uptake under water, since most stomata were closed under water, as checked with cryo SEM (Fig. 1g,f). Internal resistance for diffusion in the mesophyll is considered to be negligible, as compared to the strong barrier formed by the cuticle.

Chloroplast orientation as evidence for epidermal diffusion of CO_2

The marked orientation of the chloroplasts of the aquatic leaves provides evidence for the importance of gas diffusion through the epidermis and its cuticle layer under water. The chloroplasts of the terrestrial leaves of *R. palustris* were orientated around the intercellular air spaces and absent from walls adjoining neighboring cells (Fig. 1c), which is a universal phenomenon (Psaras et al. 1996). In contrast, the chloroplasts of the aquatic leaves were directed towards the adaxial and abaxial epidermal layers and positioned against cell walls that do not border intercellular spaces (Fig. 1d). This effect of submergence on chloroplast orientation has also been described for the amphibious species *Ranunculus flabellaris* (Bruni et al. 1996), but could not be detected in *Mentha aquatica* (Sand-Jensen & Frost-Christensen 1999).

A factor known to regulate chloroplast movement is light intensity (Wada et al. 2003; Kondo et al. 2004), but here the differential orientation of chloroplasts in the leaf types can not be caused by this factor, since light intensities were similar in both treatments. We, therefore, suggest this to be an orientation towards CO_2 supply, as has been proposed for the chloroplast orientation around the CO_2 containing intercellular air spaces in terrestrial plants (Evans & Loreto 2000). In terrestrial leaves, CO_2 enters through the stomata and then readily diffuses through intercellular airspaces, finally reaching the chloroplast. The epidermal orientation of the chloroplasts in the aquatic leaves would, from this perspective, indicate the entire epidermal layer to be the principle site of entry for CO_2 under water, with the cuticle layer being the major diffusion barrier. Interestingly, in true aquatic macrophytes, chloroplasts even develop in the epidermal cells (Rascio et al. 1999) even closer to the CO_2 source.

The clustering of chloroplasts in aquatic leaves might have decreased light absorptance (Evans & Vogelmann 2003), thus causing a slight overestimation of electron transport J_F when the terrestrial chloroplast orientation would be reestablished quickly in air (see equation 2). The partitioning of electrons to photorespiration would than be slightly overestimated, but we consider this effect to be subordinate to the other differences between the two leaf types.

Reduced diffusion resistance results in decreased photorespiration rates

Indications for the occurrence of high photorespiration under water have been derived in the past from CO_2 compensation points (Van et al. 1976; Lloyd et al. 1977; Salvucci & Bowes 1981), combined with *in vitro* determinations of the activity of enzymes from the photorespiratory and C_4 metabolism pathways (Van et al. 1976; Salvucci & Bowes 1982; Salvucci & Bowes 1983; Spencer et al. 1996). Here, we estimate *in vivo* photorespiration rate in plants under water by using gas exchange and chlorophyll fluorescence.

The combination of underwater gas exchange and chlorophyll fluorescence measurements showed that photorespiration rates can be very high under water, since the difference between A_g (sum of A_n and R_L) and J_F was up to 80 and 50 % of the total electron transport (J_F) in terrestrial and aquatic leaves at 250 μM CO_2, respectively (Fig. 4a). The relative partitioning of electron transport to photorespiration was higher in terrestrial leaves compared to aquatic leaves under water and, therefore, we conclude that acclimation to submergence considerably reduces photorespiration. The actual high photorespiratory carbon losses in submerged terrestrial leaves, however, will be limited. Respired CO_2 will be refixated immediately in a system, where diffusion of CO_2 from the water column into the leaf is rate limiting leaving other processes of reduced importance for net CO_2 assimilation.

Experimental evidence from measurements with oxygen micro electrodes showed that the aquatic leaves have a 60 % higher internal oxygen concentration than the terrestrial leaves at 250 μM CO_2 (Mommer et al. 2004). The observed higher underwater photosynthesis in aquatic leaves can thus only be explained by the much higher CO_2 diffusion into these leaves relative to terrestrial leaves, which leads to increased net assimilation and reduced CO_2 compensation point, and consequently reduced photorespiration. We thus suggest that acclimation to submergence mainly consists of an enhanced influx of CO_2 rather than leading to a reduced build-up of internal oxygen.

An assumption in these estimates of photorespiration is that the proportion of J partitioned to alternative electron sinks is similar in the different conditions. If any, the most likely change in de relative contribution of underwater electron sinks would be an increase of the Mehler ascorbate peroxidase pathway (water-water cycle), reducing O_2 directly at PSI. However, evidence for the Mehler ascorbate peroxidase pathway playing a substantial role in terrestrial plants and algae is controversial (Badger et al. 2000; Foyer & Noctor 2000; Ort & Baker 2002). Direct proof is lacking (Asada 1999; Ruuska et al. 2000; Heber 2002) and the only measurements which may imply an altered sink under water originate from measurements on drought-acclimated plants (Biehler & Fock 1996).

The excitation pressure of the electron transport system depends on the amount of electrons that can not be directed to photosynthetic electron transport or heat dissipation (Niyogi 2000), i.e. high excess energy (E, Fig. 5a) or high non-photochemical quenching (q_N

in Table 3). The proportion potentially damaging energy was relatively high in the terrestrial leaves at the lowest CO_2 concentrations under water compared to aquatic leaves, since the proportions of photons directed to photosynthetic electron transport (P) was low and safe heat dissipation (D) was probably operating at maximum capacity. In the aquatic leaves higher proportions of energy could be used in photosynthesis and photorespiration (high P), leaving less energy for D or E. Acclimation to submergence thus apparently reduces the pressure on electron transport chain, thereby reducing the potential for photodamage.

Submergence affects the ratio between carboxylation and electron transport capacity

If there has been any biochemical limitation for CO_2 affinity under water, it will have been in the aquatic leaves rather than in the terrestrial leaves, since in the first Rubisco contents were lower (Table 2) and underwater photosynthesis rates were higher (Fig. 3a). This again shows the limitation of CO_2 supply under water and thus also the importance of reduced gas diffusion resistance.

The reduced Rubisco contents in the aquatic compared to the terrestrial leaves of *R. palustris per se* were not surprising (Table 2), since this has been observed before in some, but not all amphibious plants investigated so far (Farmer et al. 1986; Beer et al. 1991). Entirely novel, however, is the finding that J_{max} was not so much reduced. Submergence increased the ratio between J_{max} and Rubisco content more than twofold, from 40 in terrestrial to 88 in aquatic leaves (Table 2). An increase of this ratio has been reported only very recently for drought stressed plants (Kitao et al. 2003; Bota et al. 2004). However, we observed a much stronger change of this ratio. The similarity in drought and submergence treated plants is a low C_i, but it remains uncertain if these responses are convergent.

Conclusion

The orientation of the chloroplasts towards the epidermis in the aquatic leaves of *R. palustris* provided evidence for the diffusion of CO_2 through the epidermis and the cuticle layer under water. The decreased thickness and most likely also decreased resistance of the cuticle in submergence-acclimated leaves reduced the gas diffusion resistance considerably, resulting in higher underwater assimilation rates and lower underwater CO_2 compensation points. Moreover, the reduced gas diffusion resistance did also result in decreased photorespiration rates and excitation pressures in the submergence-acclimated leaves under water. The biochemical level of the photosynthetic machinery was also affected by acclimation to submergence, as both Rubisco content and electron transport capacity were reduced in the submergence-acclimated leaves. However, these two factors did not change to the same extent and the data suggest an increase of the ratio between electron transport and carboxylation capacity. Acclimation to submergence thus largely consists of a reduced gas

diffusion resistance, leading to an increased influx of CO_2 and thus higher net underwater assimilation rates, a reduced fraction of photorespiration and reduced excitation pressure on the electron transport chain.

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Chapter 6

Summarising discussion

Survival and growth of terrestrial plants is negatively affected by flooding (van Eck et al. 2004). This is mainly the result of hampered gas exchange between plants and environment, since diffusion coefficients of gasses are 10^4 times lower in water than in air (Armstrong 1979). The severely limited gas diffusion between plant tissue and the environment results in oxygen deficiency and concomitantly, in energy deficits. The effects of the reduced gas exchange under water were illustrated clearly by Rijnders et al. (2000), who showed that internal oxygen pressures decrease rapidly when plants are submerged. Next to limiting gas exchange between the plant and the environment, submergence also reduces the light availability because of the high sediment load of the floodwater (Vervuren et al. 2003). This predicts low potentials for underwater photosynthesis in submerged terrestrial plants. Contrary to this prediction, even a limited amount of light strongly increases underwater plant survival (Chapter 2; Vervuren et al. 1999, He et al. 1999, Nabben et al. 1999), suggesting considerable underwater photosynthesis rates. This apparent contradiction might be explained by the hypothesis that plants are able to increase their under water gas exchange capacity.

This thesis, therefore, aimed to investigate in depth the underwater gas exchange capacity of terrestrial plants in response to submergence, and more specifically their potentials for underwater photosynthesis. The previous chapters focused on understanding the traits that are relevant for gas exchange underwater, and thus for flooding tolerance. Also the effects of light on these traits were studied, as well as the extent to which low light and submergence induce similar responses. As these experiments established the importance of gas exchange under water, further experiments aimed to increase our understanding about the consequences of the acclimation to submergence for underwater photosynthesis, in particular for photorespiration and photoinhibition.

Acclimation to submergence increases gas exchange

Several plant species are able to withstand the stresses which arise by flooding. The flooding tolerance of these species is considered to result from a suite of traits such as anaerobic fermentative pathways (Greenway and Gibbs 2003) or the elongation of petioles to restore contact with the atmosphere and thus aerial gas exchange (Voesenek et al. 2004). Testing

the adaptive value of traits has typically focused on one trait in a single species or occasionally in a wider range of taxa (Setter and Laureles 1996, Voesenek et al. 2004, Blom and Voesenek 1996), ignoring phenotypic or genetic relationships with other traits. This thesis aimed to test the adaptive value of a number of putatively important traits for flooding tolerance, by investigating how the various ecophysiological traits are related to each other and to fitness (Chapter 2). By comparing species pairs from seven taxa with one species from dry and one from regularly flooded habitats, it appeared that flooding tolerance was related to habitat of origin, rather than to phylogeny, since the flooding tolerant species constitutively expressed traits facilitating gas exchange more than intolerant species. Flooding tolerant species also showed a higher plasticity in these traits in response to submergence, compared to intolerant species. Certain traits were correlated, which suggests that coevolution of traits has occurred. In particular, coevolution may have occurred between plant height and aerenchyma content of the petiole, probably as a result of biophysical constraints. In contrast with observations in terrestrial systems (Wright et al. 2004), specific leaf area (SLA) was positively correlated with leaf longevity under water, and as such an important determinant of plant performance under water (Chapter 2).

Acclimation responses to submergence bear striking similarities with responses of terrestrial species to shade. Also aquatic plant studies repeatedly suggested that submerged leaves might in fact be inherently shade leaves (Bowes and Salvucci 1989, Boeger and Poulson 2003). This thesis shows that this suggestion is not totally unfounded, since morphological and biochemical responses to low light and submergence are in the same direction, i.e. elongation of petioles, higher SLA and higher chlorophyll a/b ratio (Chapter 3; Björkman (1981)). Moreover, underwater light compensation points also decreased in response to both shade and submergence. Maximum underwater photosynthesis rate, however, did discriminate between the functionality of the responses, as the acclimation to submergence showed to be more effective than acclimation to shade at saturating light. Underwater maximum photosynthesis rates increased stronger upon submergence than in shade, despite the fact that the increase of SLA in the shade treatment was stronger than under submerged conditions. This suggests that acclimation to submergence is a different process than acclimation to shade, and is more directed towards increased gas exchange capacity than to light capture.

Consequences of increased gas exchange for underwater photosynthesis

Direct evidence for an increased underwater gas exchange capacity upon acclimation to submergence originates from oxygen micro electrode studies on *Rumex palustris* in Chapter 4. The internal oxygen pressures were higher in submergence-acclimated plants compared

to non-acclimated plants in dark, when only passive diffusion of oxygen from the water column into the plant takes place. The higher internal oxygen pressures in acclimated plants are probably due to a reduction of the diffusional resistance for gases in the leaves. The oxygen micro electrode study also showed that the constitutive gas exchange capacity was not as severely hampered as reported in the past (Rijnders et al. 2000, Stünzi and Kende 1989), since oxygen levels did not drop to dramatically low levels in submerged plants (Chapter 4). The apparent contradiction can be explained by a difference in the thickness of boundary layers between the studies. The present study shows that if the water is not completely stagnant, oxygen pressures in both acclimated and non-acclimated shoots will be sufficiently high to maintain aerobic metabolism. Not only oxygen diffusion was facilitated in acclimated plants, but also CO_2, since in the presence of light, internal oxygen concentrations rose in these plants already at very low, but ecologically relevant CO_2 concentrations.

The orientation of the chloroplasts towards the epidermis in the submergence-acclimated leaves, instead of the terrestrial organization around the intercellular airspaces, is an indication that CO_2 diffuses through the cuticle and epidermis under water (Chapter 5). The reduced diffusion resistance in acclimated leaves of *R. palustris* is, therefore, the result from the reduced cuticle and epidermal cell wall thickness (Chapter 5). Frost-Christensen et al. (2003) showed that such change in cuticle thickness within a species is always accompanied by a more than proportional reduction of the diffusion resistance for gases. The reduced gas diffusional resistance resulted in higher underwater photosynthesis rates, higher CO_2 uptake efficiency and lower underwater CO_2 compensation points (Chapter 5), traits that will enhance benefits of light under water and therefore plant survival (Chapter 2). In addition, acclimation to submergence resulted in decreased photorespiration rates as compared to photosynthesis rates.

Under water, the availability of electron acceptors for the energy of the absorbed photons is relatively low because internal CO_2 and O_2 concentrations are low (Chapter 4). The availability of these electron acceptors increased by acclimation to submergence, due to reduced diffusion resistance (Chapter 4 & 5), which might prevent photoinhibition. The photosynthetic machinery itself was also affected as a higher investment in electron transport capacity relative to carboxylation capacity was observed in acclimated leaves compared to non-acclimated leaves (Chapter 5). It is yet unknown if this novel phenomenon is functional for submerged plants, but it might prevent damage of the photosystems.

Synthesis

This thesis shows that under water gas exchange is increased as result of acclimation to submergence, which results in higher underwater photosynthesis rates in the presence of light. All data point to a decreased gas diffusion resistance, but the magnitude of this decrease remained to be quantified. Here, I calculate the effect of acclimation on gas diffusion resistance in the flooding tolerant species *Rumex palustris*, based on the measurements of Chapter 4 and 5. This calculation of the difference in diffusion resistance in

Table 1: Conductance for diffusion of gases (g_{leaf}) of terrestrial and aquatic leaves of *Rumex palustris* in air and under water and the parameters needed for its calculation. C_a and C_c are CO_2 partial pressure in air and chloroplast respectively, $p_i[O_2]$ is internal oxygen pressure, Γ^* is the CO_2 compensation point at the defined $p_i[O_2]$, A_n is net assimilation rate at C_a.

	Aerial measurements		***Underwater measurements***	
Leaf type	**Terrestrial**	**Aquatic**	**Terrestrial**	**Aquatic**
C_a (Pa)	38	38	653 (250 µM)	653 (250 µM)
$p_i[O_2]$ (kPa)	21	21	16	26
Γ^* (Pa)	4.05	4.05	3.09	4.94
A_n (µmol m^{-2} s^{-1})	9.71±0.33	2.53±0.50	0.05±0.04	3.43±0.38
C_c (Pa) (1)	29.7±1.8	8.9±0.6	5.1±0.3	22.5±2.4
g_{leaf} (mmol m^{-2} s^{-1})(2)	219.2±92.0	9.04±1.95	0.014±0.003	0.552±0.064
Ratio	Aq/Ter = 0.04		Aq/Ter = 38	

(1) C_c was calculated for both leaf types from the model of von Caemmerer (2000), after rearrangement of equation 1 in chapter 5.

$$C_c = \frac{\left[J + 8\left(A_n + R_d\right)\right]}{\left[J - 4\left(A_n + R_d\right)\right]}\Gamma^* \qquad (1)$$

In ambient air, at 21 kPa O_2, a value of 4.05 Pa for Γ^* was used as estimated by von Caemmerer et al. (1994). Estimates of Γ^* under water are based on this value and the oxygen micro electrode measurements in the petiole (Chapter 4) since the CO_2 compensation point is proportional to oxygen concentration. Submergence acclimated leaves have higher internal oxygen pressures than non-acclimated leaves, and thus also higher CO_2 compensation points (Table 1), due to competition for binding sites at the Rubisco enzyme between O_2 and CO_2. Values of electron transport rate (J), net assimilation rate (A_n) and dark respiration rate (R_d) are available for both leaf types of *Rumex palustris* in both air and under water, as described in Chapter 5. A general assumption by applying equation 1 is that the proportion of J partitioned to alternative electron sinks is similar in the different conditions, which is discussed extensively in Chapter 5.

(2) Total leaf conductance (g_{leaf}) is proportional to the rate of assimilation and is expressed according to Fick's law (P is atmospheric pressure in Pa; Harley et al. 1992, Long and Bernacchi 2003).

$$g_{leaf} = \frac{A_n P}{C_a - C_c} \qquad (2)$$

R. palustris is used as an example to show the importance of the development of acclimated leaves under water as takes place in all tolerant species investigated. These leaves have shown to be crucial for plant performance under water, and moreover, for the impact of light on survival under submerged conditions.

Quantification of diffusion resistance of terrestrial and aquatic leaves

In air, CO_2 enters the leaf via the stomata, diffuses through the stomata into intercellular spaces and finally reaches the chloroplasts. Calculation of the total leaf resistance is, therefore, the combined result of diffusional resistances over the boundary layer, stomata, and mesophyll (Long and Bernacchi 2003, Harley et al. 1992). Under water, CO_2 diffuses through cuticle and epidermis cells to the chloroplasts, which are orientated towards these cells (Chapter 5). An additional resistance under water is the cuticle resistance (Chapter 5; Frost-Christensen et al. (2003)).

Calculations of total leaf resistance, based on measurements on *R. palustris* show that the diffusion path under water has a 15,000 times higher resistance for CO_2 than in air in terrestrial leaves (see Table 1), illustrating the tremendous difficulties for underwater gas exchange in terrestrial plants. Acclimation of *R. palustris* to submergence led to a 38 times lower diffusion resistance for CO_2 under water (Table 1). As a result, the 15,000 times higher diffusion resistance under water than in air in non-acclimated plants is reduced to a factor 400 in submergence-acclimated leaves. Thus, acclimation to submergence considerably increases underwater gas exchange capacity in *R. palustris*, and its consequences have been shown in Chapter 5. In air, terrestrial leaves have a 25 times lower diffusion resistance than aquatic leaves, which is probably due do increased stomatal resistance in the aquatic leaves in response to de-submergence.

These calculations show the enhanced gas exchange capacity upon acclimation to submergence as an example of adaptive phenotypic plasticity, as the phenotypic response is beneficial in one environment (under water), but disadvantageous in the other (air; Dudley and Schmitt 1996). This has already been recognized for heterophylly in aquatic species (Wells and Pigliucci 2000, Winn 1999), but this can now be extended to underwater acclimation in terrestrial species.

Importance of the development of aquatic leaves

As stated above, acclimation to submergence increases under water gas exchange capacity almost 40-fold. In order to acclimate successfully with respect to gas exchange it is probably required to develop new leaves as the morphological and anatomical changes may be too fundamental to take place in leaves that were already formed.

In general, the flooding tolerant species continued to develop new leaves during submergence, whereas flooding intolerant species, such as *Daucus carota* were hardly able to develop new leaves under water (Fig. 1a). This inability of flooding intolerant species is probably related to shortage of energy, as illustrated by van Eck et al. (2005), who showed that intolerant species like *D. carota* were unable to access stored carbohydrates in the taproot. Furthermore, internal aeration in these species may be poor and thus limiting

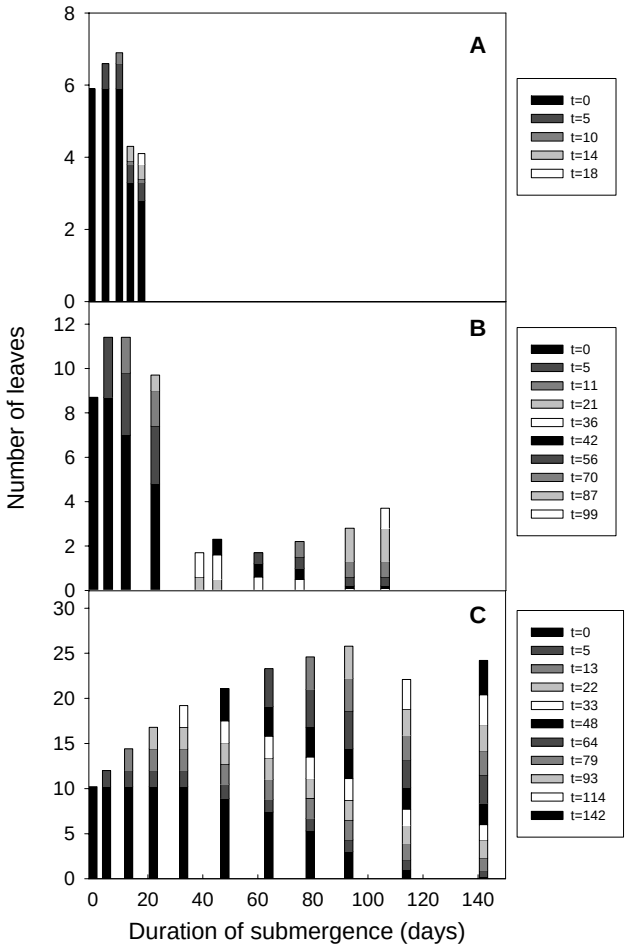

Figure 1a-c. Underwater leaf development and turnover in 3 species with different flooding tolerance. a. *Daucus carota*, b. *Rumex palustris*, C. *Mentha aquatica*. Every new stack column (color) represents a new cohort of leaves, developed under water and first present at census t (in d). The first bar (black) represent leaves present at start of submergence treatment. Experimental conditions are as in Chapter 2, and the light intensity under water is 30 µmol m^{-2} s^{-1}, data are means, n=10.

underwater plant performance.

Flooding tolerant species had different strategies of leaf formation under water. *R. palustris* showed a continuous turnover of leaves (Fig 1b), whereas other species, such as *M. aquatica*, had much lower turnover rates (Fig. 1c). The leaf morphology and anatomy of these aquatic leaves is different, as shown for *R. palustris* in Chapter 5. Another flood-tolerant species, *Oenanthe aquatica*, even developed highly dissected leaves under water, strongly resembling the submerged leaves of some aquatic heterophyllous *Ranunculus* species (Rascio et al. 1999, Garbey et al. 2004, Germ and Gaberscik 2003, Bruni et al. 1996).

Aquatic leaf type formation in heterophyllous amphibious plants has been suggested to be regulated by the plant hormones ethylene and abscisic acid (ABA) (Minorsky 2003, Kuwabara et al. 2001). Leaves grew more narrow and contained fewer stomata when *Ludwigia arcuata* was submerged or when treated with ethylene (Kuwabara et al. 2003). However, the ethylene concentrations needed to fully mimic the submergence response were exceptionally high (> 50 µL L^{-1}) and may not be reached under submerged conditions (given the data provided in the same paper, and the concentrations of 4-5 µL L^{-1} ethylene in submerged *Rumex* plants found by Voesenek et al. (1993a)). Exogenous supply of ABA was able to counteract the submergence response and switched on terrestrial leaf formation in *Marsilea quadrifolia* (Lin and Yang 1999) and *Ludwigia arcuata* (Kuwabara et al. 2003). Hsu et al. (2001) confirm these results and, moreover, showed that the ABA response was correlated with a differential expression pattern of ABA-induced ABRH genes in *M. quadrifolia*.

Contrasting evidence for ABA action originates from work on *Egeria densa*, where application of exogenous ABA induced expression of C_4-like biochemical traits (Casati et al. 2000). Thus, although ABA induces the terrestrial leaf morphology in *Marsilea* and *Ludwigia*, it induces the 'aquatic' photosynthesis type in *Egeria*. To increase complexity even further, *Eleocharis vivipara* also showed induction of C_4-photosynthesis by ABA (Ueno 1998), but in this species the C_4 traits were expressed in air instead of under water, where it showed C_3 characteristics (Ueno 2001).

To conclude, ABA plays an important role in inducing a heterophyllous switch, but it remains difficult to extrapolate its role in leaf anatomy to its role in photosynthetic metabolism. Interestingly, ABA, in an interplay with ethylene, is also a key player in submergence-induced elongation growth responses in the terrestrial species *Rumex palustris* and rice (Voesenek et al. 2003a, Kende et al. 1998), but this work focused on petioles and internodes rather than on the leaf lamina. It therefore remains to be elucidated if these hormonal interactions also cause the reduced gas diffusion resistance upon acclimation to submergence in *R. palustris*.

Benefits of light for submerged plants explained

Current literature could not relate flooding tolerance of species to underwater gas exchange capacity, since both tolerant and intolerant species showed a considerable initial underwater photosynthesis rate (Voesenek et al. 1993, He et al. 1999, Vervuren et al. 1999). These measurements, however, were performed at saturating CO_2 concentrations and do not take into account CO_2 uptake efficiency which is important under naturally low CO_2 conditions. This thesis shows that the beneficial effects of acclimation to submergence are particularly large under these low CO_2 conditions.

As a result of decreased gas diffusion resistance in the lamina, CO_2 compensation points were lower and CO_2 affinity higher in submergence-acclimated *R. palustris* plants compared to non-acclimated plants (Chapter 5), resulting in better photosynthetic performance at the naturally low CO_2 concentrations in the water column. The underwater light compensation point of *R. palustris* shifted upon acclimation from 14 to 4 µmol photons m^{-2} s^{-1} (Chapter 3), indicating that lower assimilation rates and thus lower light intensities are required to reach net positive photosynthesis. This suggests that acclimation to submergence leads to more efficient use of the low light intensities that are representative for naturally occurring submergence events (Vervuren et al. 2003). It is therefore no surprise that especially the tolerant species benefit so strongly from only a limited amount of light (30 µmol m^{-2} s^{-1}), since these species showed stronger acclimation responses to submergence than did the intolerant species. Interestingly, enhancing gas exchange capacity during acclimation to submergence will benefit both internal oxygen and CO_2 concentrations. As the CO_2 concentration determines the photosynthesis rates in light, the traits enhancing gas exchange capacity will inherently enhance the beneficial effect of light through photosynthetical carbohydrate production.

Summarising, many flooding tolerant plants were able to develop new and submergence-acclimated leaves under water, and the *R. palustris* data show that these aquatic leaves facilitate under water gas exchange. This suggests that the improvement of gas exchange capacity upon acclimation to submergence in terrestrial plants may be a general and important phenomenon in response to submergence.

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Samenvatting

Overstromingen hebben een desastreus effect op de overleving van planten die groeien in de uiterwaarden. Als het water na een overstroming weer zakt, zijn veel soorten weggevaagd of op zijn minst ten dele afgestorven (zie Fig. 1). Het grootste probleem dat een overstroomde landplant heeft, is een tekort aan zuurstof. Vrijwel alle levende organismen hebben zuurstof nodig om te overleven. Omdat gassen zich veel langzamer bewegen (een factor 10.000) in water dan in lucht, is de beschikbaarheid van zuurstof voor overstroomde landplanten veel lager dan normaal. De gasuitwisseling tussen de plant en de waterlaag is daardoor ernstig gelimiteerd. Een zuurstoftekort leidt tot energiegebrek, omdat zuurstof nodig is voor een efficiënte verbranding van suikers (ademhaling). Als er een gebrek een zuurstof ontstaat, gebruikt een plant de suikers in rap tempo op met alle gevolgen vandien.

Figuur 1. De uiterwaarden in de buurt van Nijmegen na een hoogwater. De stippellijn geeft aan tot hoe hoog het water heeft gestaan. Foto: Werner van Eck.

Er zijn echter landplanten die een aantal weken of maanden onder water kunnen staan zonder het loodje te leggen. Deze overstromingstolerante plantensoorten hebben aanpassingen ontwikkeld om de zuurstofarme condities onder water te overleven. Zo kunnen sommige planten tijdelijk overgaan op een stofwisseling waarvoor minder of geen zuurstof nodig is. Een andere respons onder water is de strekking van de bladstelen en de bladeren waardoor de plant met een ‘snorkel’ ontsnapt aan het water. Een ultieme oplossing voor het zuurstoftekort en bijkomende energiegebrek onder water zou de eigen productie van zuurstof en suikers kunnen zijn – in principe kunnen alle planten dat. Bij fotosynthese worden zuurstof en suikers gevormd vanuit koolstofdioxide (CO_2) en water. De energie nodig voor dit proces wordt geleverd door licht. Helaas is deze oplossing onder water niet zo effectief als op het eerste gezicht gedacht, want er zijn twee limiterende omstandigheden: het troebele rivierwater filtert het meeste licht weg voordat het de plant bereikt en CO_2 is een gas en daardoor, zoals gezegd, beperkt beschikbaar in water.

De beschikbaarheid van een kleine hoeveelheid licht in water met een lage CO_2-concentratie leidde echter toch tot aanzienlijke verlenging van overlevingsduur van overstroomde planten ten opzichte van overstroming in het donker (zoals ook aangetoond in Hfd. 2). Dit suggereert, ondanks de limiterende omstandigheden onder water, een belangrijke rol voor fotosynthese bij overstroming.

Het belang van fotosynthese voor planten tijdens een overstroming is dus niet eenduidig. In dit proefschrift werd daarom onderzocht of de gasuitwisseling tussen plant en waterlaag net zo beperkt is als gedacht of toch voldoende voor overleving van overstroomde landplanten. Omdat planten niet helemaal passief overgeleverd zijn aan de grillen van hun omgeving en zich - tot een bepaalde hoogte - kunnen aanpassen is expliciet bestudeerd of de gasuitwisseling kan verbeteren gedurende een overstroming. De consequenties van eventueel verbeterde gasuitwisseling voor fotosynthese onder water werden in dit proefschrift in kaart gebracht. De aandacht ging voor een groot deel uit naar de eigenschappen van de plant die van belang zijn bij fotosynthese onder water en in hoeverre die eigenschappen de tolerantie van de plant voor overstroming bepalen.

Aanpassing aan overstroming verbetert gasuitwisseling onder water

De beschikbaarheid van licht bleek van groot belang voor de overleving van een breed scala aan soorten die karakteristiek zijn voor de uiterwaarden (Hfd. 2). Soorten die meestal voorkomen op de lagere delen dichtbij de rivier en dus frequenter overstroomd worden, bleken echter veel meer aan licht te hebben dan intolerante soorten, hoger en droger op de dijk. Een verklaring hiervoor werd gezocht door een aantal eigenschappen te onderzoeken waarvan verwacht werd dat ze met gasuitwisseling te maken hadden. De tolerante soorten bleken inherent over een betere set eigenschappen te beschikken om een overstroming te weerstaan, maar ook plastischer (flexibeler) te reageren op een overstroming. Twee eigenschappen bleken de overleving van een overstroomde plant in hoge mate te bepalen. Een hoog specifiek bladoppervlak (ofwel relatief veel oppervlak ten opzichte van gewicht, zoals bij dunne bladeren; afgekort als SLA) is belangrijk omdat gassen onder water via het hele bladoppervlak naar binnen diffunderen. Daarvoor is relatief veel bladoppervlak nodig is en een lage weerstand in het blad. Een hoog aerenchymgehalte (luchtkanalen) in de bladsteel is gunstig, omdat het het passieve transport van zuurstof naar de andere delen van de plant vergemakkelijkt. Het bestuderen van de samenhang van de verschillende eigenschappen bleek cruciaal voor het begrip van de verschillende componenten die overstromingstolerantie bepalen.

Figuur 2. Moeraszuring (*Rumex palustris*) in de normale landvorm (links) en na 2 weken overstroming (rechts). Foto: Dick van Aalst, Fotografie, RUN.

In de wetenschappelijke literatuur is regelmatig geopperd dat de responsen die planten vertonen bij overstroming erg lijken op die bij schaduw. Een experiment met twee zuringsoorten (moeraszuring - *Rumex palustris*, en geoorde zuring - *Rumex thyrsiflorus*) waarbij deze planten blootgesteld werden aan beide factoren - en de combinatie daarvan - laat zien dat deze vergelijking niet geheel uit de lucht gegrepen is (Hfd. 3). De morfologische en biochemische veranderingen die geïnduceerd werden door overstroming en schaduw waren hetzelfde of op zijn minst in dezelfde richting, d.w.z. langere bladstelen, dunnere bladeren en een hogere chlorofyl a/b ratio. Deze veranderingen leidden tot identieke fotosynthese-snelheden onder water, wanneer er werd gemeten bij laag licht. Het verschil in aanpassing aan de factoren kwam echter letterlijk aan het licht, toen er onderwater fotosynthesemetingen gedaan werden bij *hoge* lichtintensiteit. De maximale onderwater-fotosynthesecapaciteit was veel hoger bij de planten die waren aangepast aan het water dan bij planten die waren aangepast aan laag licht. Dit impliceert dat er op het eerste gezicht wel overeenkomsten tussen beide factoren zijn, maar ook essentiële verschillen. Aanpassingen van planten onder water lijken meer gericht op verbeteren van de gasuitwisseling dan op het verhogen van de lichtinvang.

Consequenties van verbeterde gasuitwisseling voor fotosynthese onder water

Direct bewijs voor een toegenomen gasuitwisseling na aanpassing aan overstroming komt voort uit studies met microelectrodes (Hfd. 4) aan de overstromingstolerante moeraszuring (*Rumex palustris*, zie Fig. 2). Met deze naalddunne electroden (∅ 10 μm) kan de zuurstofconcentratie willekeurig waar in een plant zeer nauwkeurig gemeten worden. De zuurstofconcentratie in de bladsteel van een aangepaste moeraszuring was aanzienlijk hoger bij een bepaalde zuurstofconcentratie in het water dan in de bladsteel van een niet-aangepaste plant. In het licht steeg de interne zuurstofconcentratie verder, wat duidt op fotosynthese. Wanneer er meer CO_2 in het water zat, was, zoals verwacht, de interne zuurstofconcentratie nog hoger.

Deze microelectrode-studies lieten ook zien dat overstroming niet altijd rampzalige consequenties heeft voor de zuurstofconcentraties in een plant, zoals eerder werd gedacht.

Wanneer er enigszins stroming is in het water - en dat is in een natuurlijke situatie al gauw het geval - duiken de concentraties niet meteen omlaag maar blijven op z'n minst in de bladeren op een voldoende hoog niveau voor de normale zuurstofbehoevende stofwisseling.

In planten die in lucht staan, reguleren huidmondjes in het blad de opname van CO_2. Onder water zijn deze huidmondjes dicht en verloopt de opname van gassen via het totale bladoppervlak, dat normaliter lucht- en waterdicht is en daardoor een heel hoge gasdiffusieweerstand heeft. De bladeren van moeraszuring die aangepast zijn aan overstroming bleken in totaal dunner te zijn, maar ook waren de cellaag en celstructuren die de weerstand voor gasopname onder water grotendeels bepalen - de cuticula en de epidermis - dunner (Fig. 1 en 2 in Hfd. 5). Bovendien lagen de chloroplasten - de celstructuren waarin de fotosynthese daadwerkelijk plaatsvindt – in het aangepaste blad zo dicht mogelijk tegen de epidermis aan. De weerstand die CO_2 moet overbruggen wanneer het gas de plant in diffundeert, is daardoor lager in aangepaste dan in niet-aangepaste bladeren. Dat dit verschil in bladmorfologie en -anatomie daadwerkelijk van belang is, blijkt uit een hogere onderwaterfotosynthese capaciteit, een hogere CO_2-opnameaffiniteit en lagere CO_2-compensatiepunten in de overstromingsaangepaste planten (Hfd. 5), eigenschappen die de overleving onder water ten goede komen. Een ander effect van een lagere diffusieweerstand in de aangepaste bladeren was een lagere fotorespiratie – een proces dat in plaats van CO_2 vastlegt zoals bij fotosynthese, CO_2 vrij maakt. De potentiële CO_2-verliezen zijn in aangepaste planten dus lager. Als er meer licht op een plant valt dan verwerkt kan worden, kan de fotosynthesemachinerie overbezet raken waardoor schade (photodamage) aan de plant aangericht kan worden. Metingen lieten zien dat kans op photodamage in aangepaste planten lager is als gevolg van de hogere interne CO_2-beschikbaarheid.

Bovendien bleek de fotosynthesemachinerie zelf in de overstromingsaangepaste bladeren iets anders van samenstelling. Er was relatief meer electronentransportcapaciteit dan carboxylatiecapaciteit. De betekenis daarvan is nog onbekend, maar dit fenomeen is tot nu toe alleen (en in veel minder sterke mate) gevonden in planten die blootgesteld zijn aan extreme droogte.

Synthese

Dit proefschrift laat zien dat gasuitwisseling in landplanten onder water aanzienlijk kan toenemen ten gevolge van aanpassing aan overstroming. De verbeterde gasuitwisseling resulteert in hogere fotosynthesesnelheden onder water, en dus tot hogere zuurstofconcentraties en suikergehaltes. De crux van de aanpassing ligt in de verlaging van

de gasdiffusieweerstand in het blad, maar de ordegrootte van die verlaging is nooit gekwantificeerd. Dat doe ik hier voor de overstromingstolerante soort *Rumex palustris*, door de micro-electrode data (Hfd. 4) te combineren met de onderwater-fotosynthesemetingen (Hfd. 5). Deze berekening illustreert het belang van de ontwikkeling van aangepaste bladeren onder water voor de gasuitwisseling en dus voor de overleving van planten onder water.

Berekening van de diffusieweerstand in bladeren van moeraszuring

De berekeningen laten zien dat de diffusieweerstand voor CO_2 ruwweg een factor 15.000 keer hoger is in water dan in lucht, wat impliceert dat een plant bij overstroming inderdaad een sterk belemmerde gasuitwisseling heeft (Tabel 1 in Hfd. 6). De nieuw gegroeide bladeren onder water hadden onder water 'maar' een factor 400 hogere gasdiffusieweerstand dan normaliter in lucht. De aanpassingen van een blad aan overstroming heeft de gasdiffusieweerstand dus 40 keer kleiner gemaakt, en dat is een aanzienlijk verschil waarvan de voordelen boven water zijn gekomen in eerdere hoofdstukken.

De overstromingsaangepaste bladeren hadden echter ook een nadeel. Wanneer ze na onderdompeling weer boven water kwamen, hadden ze een 25 keer hogere diffusieweerstand dan de normale luchtbladeren. Wanneer een plastische eigenschap in de ene omgeving een voordeel is en in de andere een nadeel dan spreken we van 'adaptieve fenotypische plasticiteit', een evolutionair principe dat we al kennen van amfibische planten zoals oeverkruid (*Littorella uniflora*) die zowel land- als waterbladeren vormen, maar dat nu dus ook kan worden doorgetrokken naar landplanten.

De groei van 'waterbladeren' bij landplanten

In het screeningexperiment van hoofdstuk 2 zagen we dat alle overstromingstolerante soorten in meer of minder mate in staat waren tot het laten groeien van nieuwe, aangepaste bladeren onder water. Bij de intolerante soorten, zoals wilde peen (*Daucus carota*) was dat niet het geval (Fig. 1a in Hfd. 6). Een mogelijke verklaring daarvoor is een energietekort voor de vorming van die bladeren, omdat suikerreserves die in de penwortel zitten niet aangesproken kunnen worden. Verder is het interne gastransport gelimiteerd, want er zijn weinig luchtkanalen aanwezig in de intolerante plantensoorten.

De modelplant van dit proefschrift, moeraszuring (*Rumex palustris*) bleef onder water nieuwe bladeren ontwikkelen, maar er stierven ook steeds bladeren af (Fig. 1b in Hfd. 6). Watermunt (*Mentha aquatica*) spande de kroon, omdat er na 100 dagen meer bladeren waren dan aan het begin van het experiment (Fig. 1c in Hfd. 6). De bladmorfologie en anatomie van deze bladeren was verschillend van de bladeren die gegroeid zijn in lucht,

zoals eerder beschreven (Hfd. 5). Het overstromingstolerante watertorkruid (*Oenanthe aquatica*) ontwikkelde onder water fijne, sterk ingesneden bladeren.

Milieuomstandigheden reguleren de vorming van het ene of het andere type blad. De verandering van lucht naar water wordt waargenomen door verschillende signaleringssystemen en een aaneenschakeling van fysiologische en moleculaire processen vertalen dit signaal naar een groeiverandering van bijvoorbeeld het blad. Het is bekend dat de plantenhormonen abscisinezuur (ABA) en ethyleen een belangrijke rol spelen in bijvoorbeeld de strekkingsprocessen van de spruit, wanneer planten overstroomd zijn. Het blijft nog de vraag of dezelfde hormonen de verandering in bladvorm bepalen. Onderzoek bij amfibische planten lijkt inderdaad in die richting te wijzen.

Zicht op effecten van licht onder water

De positieve effecten van een spoortje licht op de overleving waren van belang voor alle planten, maar overweldigend voor de tolerante soorten. Dat was verbazingwekkend, want eerdere studies hadden geen relatie aan kunnen tonen tussen fotosynthesecapaciteit en overstromingstolerantie. Deze experimenten waren echter gedaan bij erg hoge CO_2 concentraties, en dan worden de verschillen tussen soorten in gasdiffusieweerstand gemaskeerd. Dit proefschrift focust juist op die gasdiffusieweerstand en laat zien hoe sterk deze kan verlagen bij aanpassing aan overstroming.

Als een gevolg van lage gasdiffusieweerstand zijn CO_2-compensatiepunten lager, wat betekent dat de fotosynthesesnelheid bij lage, natuurlijk voorkomende CO_2-concentraties hoger is. De parameter die de efficiëntie van lichtgebruik uitdrukt, het lichtcompensatiepunt, was lager na aanpassing van moeraszuring aan overstroming, wat betekent dat lagere lichtintensiteiten nodig zijn voor een netto positieve fotosynthese. Een lager lichtcompensatiepunt is gunstig voor een plant in een situatie waar weinig licht beschikbaar is, zoals tijdens overstroming. De aanpassingen aan overstroming leiden dus tot een efficiënter licht- en CO_2-gebruik onder water. Het is bij nader inzien ook niet verbazend dat juist de tolerante soorten, die de sterkste aanpassingscapaciteit hebben, het meeste voordeel halen uit de beschikbaarheid van licht onder water. Planten blijken bepaald niet hulpeloos te zijn als ze in een benarde situatie verzeild raken, maar het beste hiervan te kunnen maken.

Dankwoord

De AIO-tijd voorbij, het gebouw is al afgebroken, het leven gaat weer verder. Maar niet voordat ik een aantal mensen hartelijk heb bedankt voor hun hulp - in alle denkbare vormen - bij het tot stand komen van dit proefschrift. Want in je eentje is wetenschap nog niet half zo leuk.

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Om specifieke fotosynthese-expertise te verkrijgen, buurtte ik regelmatig in Utrecht bij de groep Ecofysiologie van Planten, waar ik altijd bij Thijs Pons terecht kon. Thijs, onze discussies over de consequenties van fotosynthese in de onderwaterwereld leidden altijd weer tot nieuwe ideeën en inzichten, en hopelijk blijven ze dat doen. Dank voor al je nauwgezette raad en soms stellige, maar immer praktische adviezen, waardoor mijn project meer diepgang kreeg. Rens, dank voor het enthousiasme waar je me altijd mee verwelkomde in het Wentgebouw. Danny, mijn Rubisco avontuur was heel wat minder glorieus verlopen zonder je hulp: dank! Ook alle andere Utrechtse ecofysiologen wil ik bedanken voor hun practische hulp, interesse en plezier.

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De Nijmeegse afdeling Experimentele Plantenecologie was voor mijn komst een typische mannenafdeling, desalniettemin was het snel een fijne plek om te werken. Paul: dank voor je

hulp in de opstartfase. En zonder je latere hulp op afstand met de overlevingsanalyses hadden mijn planten niet zo nauwkeurig overleefd. Verder veel dank aan Werner, John, Gerard (dank voor je lessen in de mysteries van zuurstofelectroden), Harry, Ruud en Ronald. Het bier vloeide minder rijkelijk na de intocht van meer dames (Julia, Corien, Coco, Tamara, Linda, Ling, Hannie, Annemiek, Heidi) en nieuwe mannen (Josef, Jelmer en Eelke), maar de werksfeer bleef goed. En José was en is het rustpunt daarbinnen – een waardevol gegeven. Tim Kolbrink en Ron Galiart hebben als studenten binnen mijn project veel werk verzet, waarvoor mijn dank!

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Lieve Ronald, wetenschap werd inspirerend door er met jou over te filosoferen. En dat is en blijft zo. Het leven gaat verder, gelukkig samen met jou! Ik hou van je!

List of publications

Mommer L, Pedersen O, Visser EJW. **2004.** Acclimation of a terrestrial plant to submergence facilitates gas exchange under water. *Plant Cell and Environment* **27:** 1281-1287.

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Curriculum vitae

Liesje Mommer werd geboren op 9 februari te Heerlen. Zij begon in 1994 aan de studie biologie aan wat toen de Landbouw Universiteit Wageningen heette en studeerde in juni 2000 cum laude af aan de van naam veranderde Wageningen Universiteit. Zij had zich voornamelijk gespecialiseerd als mathematisch bioloog, door middel van een tweetal stages waarin het modelleren van gewasproductie centraal stond. Deze projecten brachten haar naar West Afrika voor studies aan cacaoteelt, en naar Zwitserland alwaar prei centraal stond. Gelukkig was er tijdens het afstudeervak bij de vakgroep Terrestrische Oecologie en Natuurbeheer ook ruimte voor experimenteel onderzoek. Dit keer aan grassen die op zoek gaan naar nutriënten. Dit laatste project maakte haar enthousiast voor experimenteel onderzoek, wat leidde tot een aanstelling als Junior Onderzoeker bij Experimentele Plantenecologie aan de Radboud Universiteit Nijmegen. De resultaten van haar promotieonderzoek over onderwaterfotosynthese van landplanten staan in dit proefschrift beschreven en werden op diverse symposia gepresenteerd. Voor haar promotiewerk werkte ze uitgebreid samen met diverse onderzoeksgroepen, wat leidde tot werkbezoeken aan dr. Ole Pedersen in Denemarken, prof. dr. Theo Elzenga en dr. Jan Henk Venema te Groningen en dr. Thijs Pons in Utrecht. Zij was naast haar onderzoek ook betrokken bij het begeleiden van studenten tijdens hun stages, de Alpenexcursie van de Nijmeegse plantenecologen en de ecofysiologiecursus. Tevens verzorgde zij gastcolleges aan de Universiteit Utrecht.
Sinds oktober 2004 is Liesje als post-doc werkzaam bij de leerstoelgroep Experimentele Plantenecologie. De aandacht verschuift daarin van onderwater naar ondergronds: van een onderwerp waar licht de sleutel is tot een onderwerp waar daglicht niet wordt verdragen.